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THE
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PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

A QUANTITATIVE STUDY OF PHENOCOPY PRODUCTION WITH MONOCHROMATIC ULTRAVIOLET IRRADIATION

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The term phenocopy was introduced by Goldschmidt (1935) to refer to forms, produced by some experimental procedure, whose appearance duplicates or copies the phenotype of some mutant or combination of mutants. The first experiments with phenocopy production were those of Standfuss (1896) who, by treating butterfly pupae with high or low temperatures, produced adults which resembled other geographic races. Phenocopies have been produced in Drosophila by high temperature (Goldschmidt, 1929, 1935; Plough and Ives, 1932, 1935; Child, Blanc, and Plough, 1940), low temperature (Gottschewski, 1934), X-rays (Friesen, 1936; Waddington, 1942; Villee, 1946a), chemical agents (Rapoport, 1939), visible light (Villee and Lavin, 1946), and ultraviolet light (Geigy, 1931; Eloff, 1939; Epsteins, 1939). Geigy irradiated only very early egg stages and obtained flies with abnormal abdomens, legs, and wings. Eloff was interested primarily in the effects of ultraviolet light on crossing-over but observed some wing abnormalities when late pupae were irradiated. Epsteins irradiated larvae and pupae and produced abnormal abdomen, hemithorax, the absence of the scutellum, and abnormalities in the wings, chiefly scalloping of the distal and posterior edges of the wings. In none of these experiments was the intensity of the ultraviolet light measured.

This study was undertaken to provide quantitative data of the effects of ultraviolet light on larval and pupal stages. It was originally planned to use both 2537 A and 2800 A light to see if the phenocopy-producing reactions could be ascribed to changes in nucleic acid or protein metabolism. However, a light source providing 2800 A light could not be obtained so experiments with that have been postponed. Davis (1944) made a quantitative study of the effects of ultraviolet in inhibiting the folding process in neural tube formation in chicks. By using monochromatic light of different wave-lengths obtained from a monochromator he determined the photochemical efficiency curve for the process. This was found to compare closely with the absorption curve of sterols, especially that of 7-dehydro-

[1] I am greatly indebted to Dr. George I. Lavin for supplying the ultraviolet lamp, filter, and meter used in these experiments and for furnishing much invaluable advice and criticism during the course of the study, and to Dr. Eric G. Ball for the facilities of his laboratory.

cholesterol, with maxima at 2576 A and 2804 A. Schechtman (1944) found that the inhibiting effects of ultraviolet light on the development of Hyla eggs were slightly stronger at 2537 A than at other bands tested and Landen and Uber (1939) found that the inactivation of yeast by ultraviolet was greatest at 2600 A, where 500 ergs/mm.^{2} produced 50 per cent inactivation. Stadler and Uber (1942) found that the photochemical efficiency curve of ultraviolet light in producing mutations in maize corresponded to the absorption curve of nucleic acids and Hollaender (1945) and colleagues found that ultraviolet of wave-length 2600 A was much more efficient than other wave-lengths in producing mutations in a variety of fungi, Neurospora, Trichophyton, Penicillium, and Aspergillus.

Materials and Methods

Three stocks were irradiated: a wild type and an aristapedia-Bridges (ss^{aB}, chromosome 3, locus 58.8) stock isogenic with it (Villee, 1946b) and an independent, miniature wing (m, chromosome 1, locus 36.1) stock. Larvae were obtained by allowing large numbers of stock flies to lay eggs for a two-hour period on corn meal-molasses-agar food in half-pint bottles. The ages of the larvae are thus known to within ± one hour. The cultures were kept at 25.5° C. before and after irradiation. At this temperature the larvae pupate within a few hours of 100 hours after they hatch from the eggs. Prepupae (white pupae) were collected frequently and the time noted. The age of the pupae at irradiation was determined from this. A total of 3,500 flies was irradiated in groups of 25 larvae or pupae.

The source of the ultraviolet radiation was a spiral quartz mercury resonance lamp, manufactured by the Hanovia Chemical Company, which emits about 80 per cent of its energy in the form of the 2537 A line. A 120 mA. luminous tube transformer was used. The visible radiation was removed by a quartz filter cell containing a mixture of nickel sulfate and cobalt sulfate dissolved in distilled water (Backström, 1940). The lamp used had been burned well over 100 hours before the experiments began so the amount of radiation of wave-lengths shorter than 2537 A should be negligible. The intensity of the irradiation was varied by altering the distance between the lamp and the target. The intensity was measured by a Hanovia ultraviolet meter, the target of which was fastened to a carriage on an optical bench. The larvae or pupae to be irradiated were placed in a small uncovered Petri dish on this carriage. By moving the carriage back and forth, intensity measurements were taken before and after each irradiation and always checked very closely.

The larvae to be irradiated were removed from the culture bottles, washed briefly in 70 per cent alcohol, rinsed in Ringer's solution, dried on filter paper and placed in small, uncovered, dry Petri dishes. The larvae very shortly became stuck to the glass and showed no tendency to escape from the dish. After irradiation, the larvae were moistened with Ringer's solution to free them and removed with a camel's hair brush to shell vials containing culture medium to complete development. This drying treatment had no deleterious effect on the larvae: several groups of larvae were handled in this way and dried 20 to 30 minutes without irradiation and all hatched out normally.

Results

Larvae show a gradual increase in sensitivity to ultraviolet radiation with age from 50 to 100 hours after hatching to a maximum at one hour after pupation, then a sharp decrease in sensitivity with pupal age. The sensitivity of flies to ultraviolet at an intensity of 44 ergs per mm.^{2} per second for different durations of exposure, as measured by the percentages killed, is given in Figure 1.

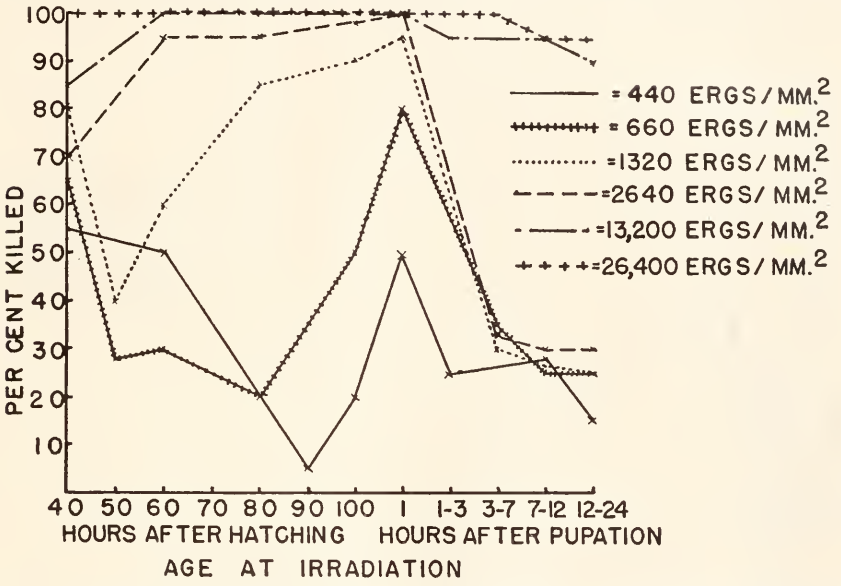

Figure 1. Percentage of flies killed by different total dosages of irradiation at different ages given at 44 ergs/mm.^{2}/sec.

The percentage of flies showing phenocopies also varies with the age of the larva or pupa irradiated (Fig. 2). There seem to be two periods during the larval stage of greater phenocopy production for a given amount of radiation, one about 50 and one about 100 hours after the larvae hatch from the egg. The entire larval period from 40 to 100 hours after hatching is one of fairly high phenocopy production, higher than the first 24 hours of pupal life. Some pupae showed very high percentages of phenocopies, up to 400 per cent (400 phenocopies per 100 flies), but this was caused by the fact that the pupae can withstand more energy for the production of phenocopies without being killed. The high percentages of phenocopies were the result of irradiations with 13,200 or 26,400 ergs/mm.^{2} Since these dosages killed 100 per cent of the flies irradiated at most of the ages used, no lines were drawn for them on Figure 2. In 7- to 12-hour pupae, irradiations of 3,960 ergs/mm.^{2} gave 120 phenocopies per 100 flies, 13,200 ergs/mm.^{2}, 200 phenocopies per 100 flies, and 26,400 ergs/mm.^{2}, 300 phenocopies per 100 flies.

The phenocopies produced included abnormal abdomen, combgap legs, abnormal thorax, small or rough eye, fused eye facets, folded, dumpy, curled or balloon wings, abnormalities in the wing veins, fused, singed or missing bristles and microchaetes,

doubled sex combs on a male prothoracic leg, and a shoulder-like protrusion growing anteriorly from the mesothorax.

The most numerous type of phenocopy produced was that involving an abnormality in the abdomen, some deformity in or the complete absence of one or more tergites. These were produced by irradiating flies in any stage of development from 40-hour larvae to 24-hour pupae. Geigy (1931) produced similar phenocopies by irradiating eggs ½ to 17½ hours old. (See Geigy for figures of the variations in phenotype produced.) Epsteins (1939) reported similar abnormali-

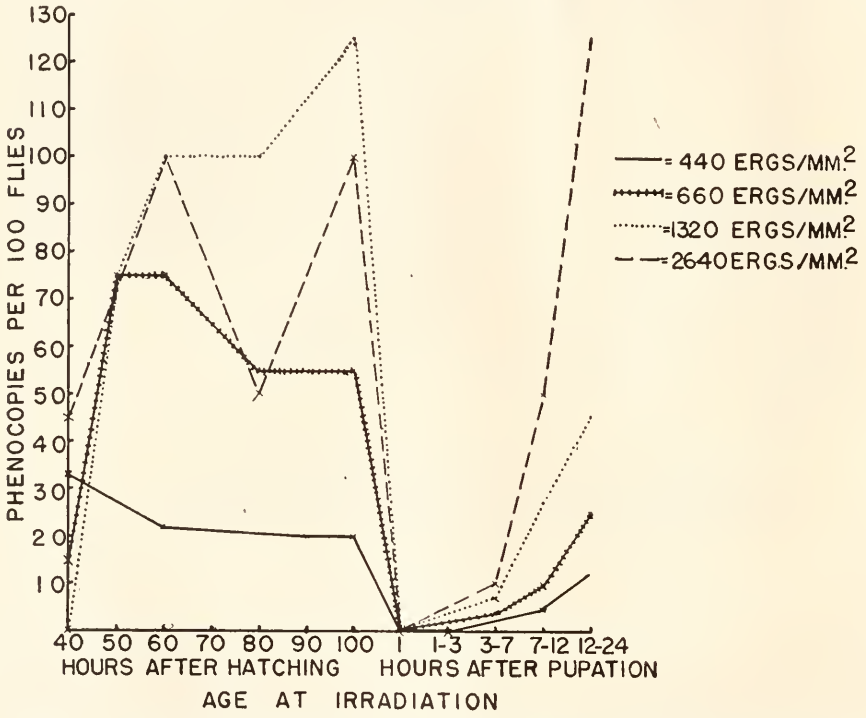

FIGURE 2. Number of phenocopies per 100 flies produced by different total dosages of irradiation at different ages given at 44 ergs/mm.²/sec.

ties produced by irradiations at times comparable to those used in this study. The large numbers of these resulting from irradiations during either the egg, larval, or early pupal stage is probably due to the fact that the dorsal abdominal imaginal discs lie close to the dorsal surface and are more readily reached by the rays than are deeper-lying discs. Eloff (1939) showed that ultraviolet light penetrates one thickness (about 10 microns) of Drosophila cuticle with only slight diminution of effect, but that four thicknesses screen out the effective rays.

Abnormalities in the formation of the mesothorax and scutellum occur after irradiation during the pupal period, especially between 7 to 12 and 12 to 24 hours after pupation. These include absence of the scutellum, failure of the right and left dorsal mesothoracic discs to fuse, failure of one disc to unfold properly, etc., giving phenotypes similar to those of certain grades of tetraltera (see Villee, 1942,

for figures). The eye phenocopies, small, rough, or fused eye facets, also were obtained after irradiation in the pupal period, especially between 7 and 24 hours after pupation. One fly irradiated in this period showed large, swollen legs resembling the phenotype of combgap. The wing and bristle phenocopies were produced primarily by irradiation in the pupal period, 7–24 hours after pupation. Reduction of the microchaetes occurred only after irradiation in this period. An additional period in which both bristle and wing phenocopies were produced was between 80 and 90 hours of larval life. Dumpy wings were produced only by the irradiation of the pupae.

The three stocks used in general responded similarly to irradiations of comparable developmental stages. The single exception was aristapedia-Bridges, which frequently developed 2- to 4-segmented tarsi when irradiated as 80- or 90-hour larvae or as 7- to 12-hour pupae. This same stock also develops very short, 1- to 4-segmented tarsi when treated with X-rays (Villee, 1946a), low temperature (Villee, 1943) or when combined with "growth rate" genes (Villee, 1945), although under normal conditions it always has normal 5-segmented tarsi (see Villee, 1946a for discussion).

Only two phenocopies were found which resembled the pervasive effects of overgrowth or abnormal histogenesis found frequently with X-radiation of 70- to 90-hour larvae (Waddington, 1942; Villee, 1946a). One wild type male irradiated as a 3- to 7-hour pupa with a total of 5,280 ergs/mm.^{2} developed a second pair of sex combs on the second tarsal segment of the prothoracic leg, and resembled closely a phenotype obtained with X-radiation (Villee, 1946a, Fig. 8). One wild type female irradiated 100 hours after hatching from the egg, about 2 hours before pupation, with 2,640 ergs/mm.^{2} developed a small palp-like outgrowth from the anterior dorso-lateral margin of the thorax, apparently identical with those obtained with X-radiation (Villee, 1946a, Figs. 1–3).

Irradiation of larvae with ultraviolet does not cause a retardation of pupation as X-radiation does. The larvae pupate in the normal time after ultraviolet treatment; X-radiation causes a retardation of pupation of 8 to 12 days beyond the time when controls pupate (Villee, 1946). With X-radiation there is also a marked difference in the results of irradiation of about the same dosage given at different intensities. A given dosage at high intensity causes a much higher lethality than a similar dosage at low intensity. With ultraviolet radiation, this difference is not found. A given dosage of ultraviolet light, whether given at 44, 37, 27, 17.5, or 11 ergs/mm.^{2}/sec., produces about the same percentage of lethality and of phenocopies. It may be that the range of ultraviolet intensities used was not great enough. There is only a factor of four between the lowest and highest intensities, whereas in the X-ray experiments the high intensity was 71 times the low intensity (5,540 *vs.* 78 r. units per minute). However, Carlson and Hollaender (1944) found that the effects of 2537 A light on mitosis in grasshopper neuroblasts depend simply on the total dosage and not on the intensity even when it is varied by a factor of 1500. In a later paper (Carlson and Hollaender, 1945) they found that at low total dosages (57.6 ergs/mm.^{2}) intensities varying from 0.004 to 16.3 ergs/mm.^{2}/sec. showed no significant differences in the effect on mitosis, but at high total doses (172.8 and 230.4 ergs/mm.^{2}) treatments given at high intensity were slightly more effective in depressing mitosis than ones at low intensity. The dosages used in this study were higher than Carlson and Hollaender's highest but

no intensity effect was observed. Bain and Rusch (1943) reported just the opposite conditions in the production of tumors in mice by ultraviolet radiation. They found ultraviolet of wave-lengths 2800–3400 A more effective in producing tumors when given at low intensities over long periods of time than when given at high intensities for short periods. They used much greater amounts of energy, 116–212 $\times 10^5$ ergs/mm.^{2}, than used by Carlson and Hollaender in their experiments. The high intensity, 1.35 $\times 10^5$ ergs/mm.^{2}/day, was about four times their low intensity, 0.35 $\times 10^5$ ergs/mm.^{2}/day.

Discussion

The factors regulating the production of phenocopies by temperature treatments are: (1) the developmental stage at which the treatment is applied; (2) the extensity (total time) of the treatment; (3) the intensity of the treatment; and (4) the genotype of the animals treated (Goldschmidt, 1929, 1935). With ultraviolet light, the important factors are the age of the fly at irradiation, the stock used, and the total dosage of irradiation. Within the range of intensities used in these experiments, variations in intensity had no effect in changing the percentage of lethality or of phenocopies. The phenocopies produced may, in the main, be explained as due to the absorption of the light energy in individual cells or small groups of cells, probably by the nucleic acids, since 2537 A is close to the region in which they absorb maximally, 2600 A. Since both desoxyribose nucleic acid, located in the chromosomes, and ribose nucleic acid, located in the cytoplasm as well as in the chromosomes, absorb at the same wave-length, it is impossible to decide whether the phenocopy-producing reaction is localized in the nucleus, cytoplasm, or in both. The fact that ultraviolet of 2537 A wave-length affects the chromosomes and retards mitosis in grasshopper neuroblasts (Carlson and Hollaender, 1944) would suggest that the phenocopy effect is also mediated by the chromosomes. Hollaender, Greenstein, and Jenrette (1941) found that 2537 A radiation causes a depolymerization of sodium thymonucleate (desoxyribonucleate) *in vitro*. As a working hypothesis we may suppose that irradiation of Drosophila larvae or pupae with 2537 A is absorbed by nucleic acids or nucleoproteins in the chromosomes of the cells of the imaginal discs near the surface. The absorption of this energy results in a physical change, a depolymerization, of the nucleic acid with a consequent upset in the structure of the gene so that it is partially or completely inactivated, with the result that development of that structure is abnormal. Since the inactivated genes are located in some body cell rather than a germ cell, a phenocopy rather than a mutation is produced. Future research may, of course, show that the phenocopy-producing mechanism is entirely different from that proposed here.

Some of the phenocopies found in these experiments suggest that the cells of the imaginal discs were killed, others suggest that certain genes were altered or inactivated, perhaps by the scheme outlined above. Only two of the phenocopies, a doubling of the sex combs on the male prothoracic leg, and a palp-like outgrowth from the anterior dorso-lateral margin of the thorax, each of which occurred only once in the course of the study, suggest the pervasive effects of abnormal histogenesis found frequently with X-radiation. These have been explained (Waddington, 1942; Villee, 1946a) by assuming that the X-rays cause the death of cells

and that the dead or necrotic cells release diffusible morphogenetic substances which result in the abnormal histogenesis or overgrowth. The same explanation may be applied to the abnormal histogeneses produced by ultraviolet radiation.

The sensitive periods for certain of the phenocopies are slightly different from those found for X-rays or temperature treatments. It is rather difficult to compare the work of different investigators, who use different stocks raised at different temperatures, but it would appear that the sensitive period for the production of dumpy wings by ultraviolet corresponds with that found by Blanc and Child (1940) for temperature treatments and that the larval sensitive period for bristle reduction by ultraviolet is identical with the temperature sensitive period determined by Child (1935). In addition there is a sensitive period for bristle reduction by ultraviolet in the pupal stage which was also found in X-radiation experiments (Waddington, 1942; Villee, 1946a). The sensitive period for the reduction of the size of the eye by ultraviolet is in the pupal period, 7–24 hours after pupation, whereas Goldschmidt (1935) found the temperature sensitive period to be in the larval period, at an age corresponding to approximately 90 hours after hatching from the egg. Abnormalities in the thorax appear after ultraviolet irradiation in the pupal period, 7–24 hours after pupation, but in the larval period at ages corresponding to 50–100 hours after hatching from the egg following X-radiation (Villee, 1946a). The wing phenocopies produced by ultraviolet had two sensitive periods, one in the larval period about 80–90 hours after hatching from the egg, which corresponds to the temperature sensitive period (Goldschmidt, 1935) and an additional one in the pupal period not found with temperature treatments.

The energy threshold for the production of phenocopies is slightly below the lowest total dosages, 330 and 440 ergs/mm.^{2}, used in these experiments and varies with the age of the fly irradiated. At one hour after pupation, no phenocopies were produced by 440 ergs/mm.^{2} but the dosage caused a 50 per cent mortality among the pupae irradiated. At other ages, from 5 to 33 per cent phenocopies (i.e., 5 to 33 phenocopies per 100 flies) were obtained with this dosage. This threshold level is of the same order of magnitude as that found by Landen and Uber (1939) for the inactivation of yeast (500 ergs/mm.^{2} produced a 50 per cent inactivation) and by Giese (1946) for the production of abnormalities in developing echinoderms by the irradiation of sperm before fertilization. He found the threshold level for the production of abnormalities in development by irradiating the eggs of these forms to be considerably higher, on the order of 5,000–8,000 ergs/mm.^{2} Carlson and Hollaender (1945) found a considerably lower threshold in the effects of ultraviolet on mitosis in grasshopper neuroblasts: about 100 ergs/mm.^{2} produce a reduction of the mitotic ratio to 0.5. Davis (1944) found that the energy required to inhibit the folding process in neural tube formation varied with the wave-length of the ultraviolet used and that about 200 ergs/mm.^{2} produced the inhibition when ultraviolet of 2537 A was used.

It is impossible to make an exact comparison between the actions of X-rays and ultraviolet rays per energy unit, first, because their modes of action are different, and second, because only a small percentage of the X-rays are absorbed and become effective in the tissue irradiated, the rest pass through without affecting the cells, whereas ultraviolet rays are largely absorbed by tissues as thick as a Drosophila larva or pupa. However, it is of interest to make a rough comparison between the experiments reported here and the previous study using X-rays

(Villee, 1946a). It was found that the threshold for the production of phenocopies with X-rays was slightly below 1096 r. units. At that level from 0 to 40 per cent of the flies were killed and from 0 to 10 per cent showed phenocopies. This dosage D in roentgens may be converted to the density of absorbed energy, E, by the formula $E = 83\ D$ ergs/cm.^{3} (Cole, personal communication) to give 90,968 ergs/cm.^{3} The absorbed energy of the ultraviolet radiation, computed from the area and volume of the larva and the minimal threshold intensity of 440 ergs/mm.^{2} and assuming total absorbtion by the organism, is 440,000 ergs/mm.^{3} From this it can be seen that X-radiation is approximately five times as effective per energy unit as ultraviolet radiation of wave-length 2537 A in producing phenocopies. I want to thank Professor Kenneth S. Cole for his assistance in making these calculations.

Summary

1. Drosophila larvae, prepupae and pupae of various ages and genotypes were irradiated with ultraviolet of wave-length 2537 A and in dosages varying from 330 to 79,200 ergs/mm.^{2} The phenocopies produced varied with the age of the irradiated fly, the stock used, and the total dosages of the irradiation. Irradiation with ultraviolet does not cause a retardation of pupation as X-radiation does.

2. Larvae show a gradual increase in sensitivity to ultraviolet radiation with age from 50 to 100 hours after hatching from the egg to a maximum at one hour after pupation, then a sharp decrease in sensitivity with pupal age. There are two periods in the larval stage, one about 50 and one about 100 hours after hatching, of greater phenocopy production for a given amount of radiation. Irradiations during the first 24 hours of pupal life produce fewer phenocopies for a given amount of radiation than during the larval period.

3. The sensitive periods for the production of certain phenocopies by ultraviolet are compared with the sensitive periods for X-ray and temperature treatments. Some are identical, a few are different.

4. Irradiations of the same total dosage produce the same percentages of lethality and of phenocopies whether given at high or low intensities. The threshold level for the production of phenocopies varies with the age of the fly irradiated but is about 440 ergs/mm.^{2} A comparison is made of this threshold with the thresholds for the effect of ultraviolet on other biological systems and with the effect of X-rays on phenocopy production in Drosophila.

5. A hypothesis for the mechanism involved in the production of phenocopies by ultraviolet rays is discussed.

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CYTOLOGICAL AND EXPERIMENTAL STUDIES ON THE OÖCYTES OF FRESH WATER PULMONATES[1]

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The organization of the ovum and the importance and relation of its inclusions such as yolk, oil, pigment, and other granules, to the ontogenic process are of considerable interest to embryologists.

Early centrifugation experiments on the eggs of many invertebrates established the facts that distribution of the visible elements apparently has no direct causal relation to the cleavage pattern of the egg or the development of the adult, and that there must be some sort of formed or underlying ground substance which could be only slightly affected, if at all, by centrifugal force (Lillie, 1906, 1909; Morgan, 1908; Morgan and Spooner, 1909; Conklin, 1910). More recently Raven and Bretschneider (1942), as the result of experiments with the eggs of the pond snail, *Lymnaea stagnalis,* have questioned the premise that distribution of visible elements in the egg has no effect on the developmental pattern. However, they have been unable to cause any atypicalities in ontogeny attributable to centrifugation and stratification of these elements.

Practically all of the centrifugation experiments directed at gaining information about the organization of the ovum or the role of the visible inclusions in development have been made on eggs already deposited, and, in many cases, already fertilized. Few, if any, have considered the egg from the point of view of its development prior to its release from the gonad, during the time when its organization might be considered in the "formative stage."

The present paper is a report of some cytological and experimental investigation on the ovarian oöcytes of two species of fresh water snails, *Lymnaea stagnalis appressa* Say and *Physa gracilis.* These species were chosen for investigation because their eggs exhibit a spiral type of cleavage which is determinate. Hence, they may be said to be "restricted" at a very early stage in development. Moreover, the potentialities of the deposited eggs of numerous species in the genera *Physa* and *Lymnaea* have already been thoroughly investigated and hence there has accumulated much information against which new research can be contrasted and properly evaluated.

The cultures of *Physa* were obtained from laboratory stocks on hand at the Biological Laboratories of Harvard University, while those of *Lymnaea* were imported to the laboratories from Wisconsin. Both cultures were kept in laboratory aquaria where they reproduced readily without special culture techniques other than those usually followed in maintaining balanced aquaria.

[1] The experimental work described herein was done at the Biological Laboratories, Harvard University, Cambridge, Mass.

* The writer wishes to express his sincere gratitude to Professor Leigh Hoadley for his kindness, generosity, and many helpful suggestions pertinent to the course of this work.

The body of the work may be divided into three parts. The first includes a cytological comparison of the developing oöcytes of *Lymnaea* and *Physa*. The second deals with an analysis of the effects of centrifugation upon the ovarian oöcyte, and the third is concerned with a comparison of the effects of X-radiation on the two species.

For all cytological studies, unless otherwise indicated, Champy fixing fluid was used, followed by the Champy-Kull staining procedure. The gonads were excised and then fixed, since the size of the snails made it impractical to obtain good fixation by immersion of the entire animal. Material was embedded in paraffin and sectioned at five microns.

The Developing Oöcyte

As might be expected from the relative sizes of the two species, the gonad of *L. stagnalis* is considerably larger than that of *P. gracilis*. In both species, the hermaphroditic gland is found embedded in the liver on the inner side of the coiled visceral "hump." The organ is lobed and slightly more compact in its consistency than is the surrounding liver tissue. It may be found easily by tracing the prominent white and convoluted oviduct to the point where it appears to enter the body of the liver. This point marks the anterior aspect of the gonad.

The superficial histological picture of the active ovotestis presented by the two species is essentially the same. In both one finds numerous tubules in which the growth and maturation of sperms and the development of oöcytes goes on simultaneously. The oöcytes are situated along the walls of the tubules and may be identified quite early in development by their location and by their enlarged nuclei. At the earliest recognizable stage they measure about 10–15 μ in diameter, while at the time of release from the gland the diameter has increased to approximately 130–140 μ. In spite of the difference in the size of the adults of the two species, the size of the ova is about the same. For an account of the general histology of the mature ovotestis, the reader is referred to the work of Crabb (1927) in which the species *Lymnaea stagnalis* is treated at considerable length. The present report will present only sufficient descriptive background to clarify fully the position and relations of the specific regions of the gonad under consideration.

With Champy-Kull fixation and stain, the ovarian oöcytes of both *Lymnaea* and *Physa* show evidences of a clearly marked polar differentiation by the time they have reached one-third of their maximum size. The germinal vesicle is displaced slightly toward one pole of the cell and a center of accumulation of droplets or granules which are blackened by osmic acid is present in the region of the opposite pole. Yolky granules which stain red fill the cell completely and obscure details of any other cytoplasmic inclusions which might be present. The yolk granules show a slight gradation along the axis of polar differentiation, being a little finer in the region of the germinal vesicle and coarser at the opposite end of the cell (Plate *1A* and *B*; Fig. 1*A*). A detailed study of the morphological evidences of the polarity reveals certain differences in *Physa* and *Lymnaea* which are expressed through the movements of the osmiophilic inclusions during the development of the oöcyte.

In *Lymnaea,* these black droplets appear first when the egg is about 25 μ in diameter. They arise in the vegetative region and remain there until after the

release of the oöcyte. As the oöcyte increases in size, the number of the globules in the mass increases until the vegetal pole becomes literally packed with them (Fig. 1*B*). Following shedding of the oöcyte, the osmiophilic inclusions spread out from their massed apolar position and invade the region of the red-staining yolky granules, with the result that the visible polarity begins to be obscured

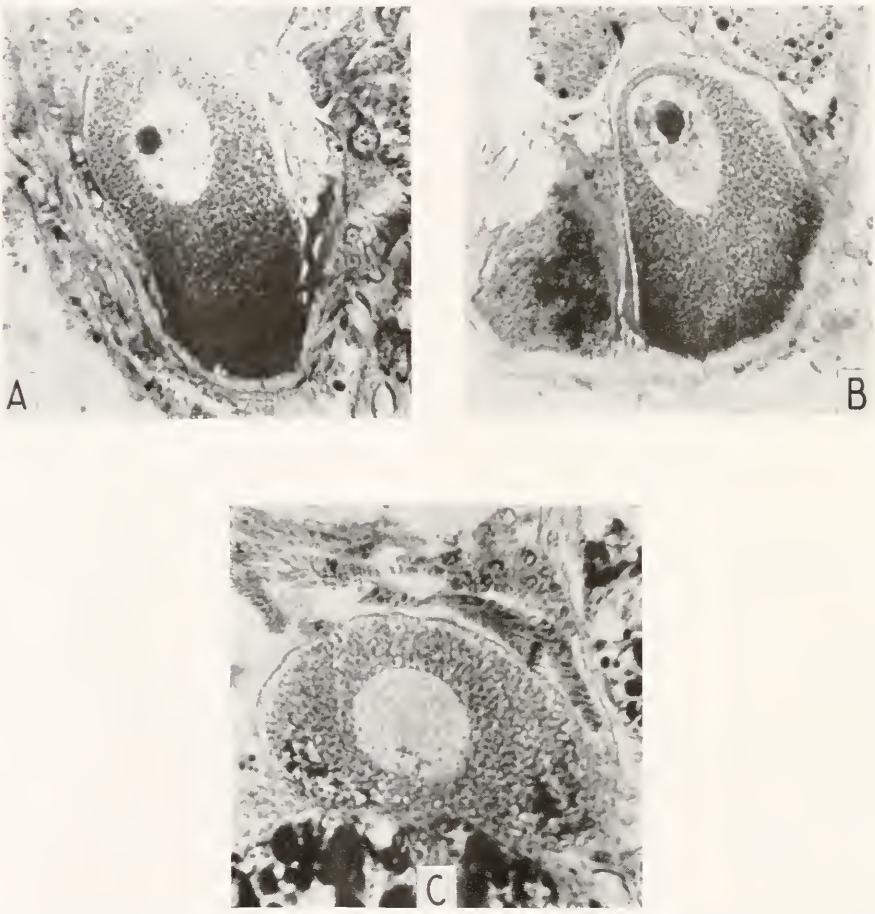

PLATE I. Photomicrographs of oöcytes within their envelopes in the hermaphroditic gland. *A* and *B*, *Lymnaea*; *C*, *Physa* (× approximately 500)

(Fig. 1*C*). This migration starts before any signs of breakdown appear within the germinal vesicle, and progresses more rapidly in the subcortical portion of the cell. With Champy-Kull stain it is difficult to determine whether this migration is the result of factors instigated solely within the oöcyte, or whether it is the result of an activation caused by fertilization. The work of Crabb (1927) on *Lymnaea* argues strongly for the latter explanation. But regardless of the cause of its origin, it is to be stressed that the pattern of migration is constant for all oöcytes, and the osmiophilic material always has the same distribution in oöcytes

at comparable stages of development. By the time the maturation process has begun and the nucleus is no longer intact or visible the osmiophilic mass appears equally distributed throughout the cell (Fig. 1*D*).

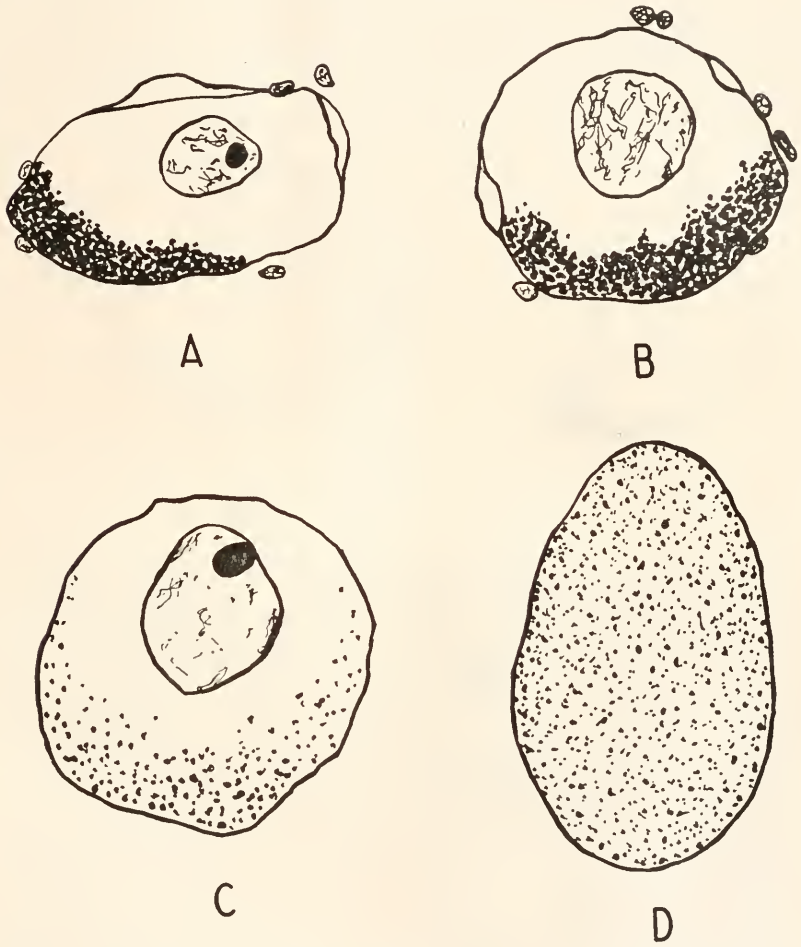

FIGURE 1. Sketches based on camera lucida tracings to show distribution of osmiophilic material during the course of development of the oöcyte of *Lymnaea*. *A* and *B*, ovarian oöcytes; *C*, oöcyte at the time of release from surrounding envelope; *D*, oöcyte in tubule of gonad just after breakdown of germinal vesicle.

In *Physa* the darkened globules appear first when the oöcyte is about 25–30 μ in diameter. Though there is some accumulation of these globules in the vegetative pole of the cell the massing is by no means as pronounced as in *Lymnaea* (Plate I*C*; Fig. 2*A*). Instead, one notes the beginning of a migration of osmiophilic material from the vegetative pole toward the animal pole even in the smaller oöcytes. As the oöcyte enlarges in its envelope the spread becomes more and more pronounced, while the massing in the vegetal pole is less pronounced. By the time the cell has attained three-fourths of its growth, the distribution of the osmio-

philic material is directly comparable with that of the released or shed oöcyte of *Lymnaea* (Fig. 2*B*).

Following the release of the oöcyte of *Physa* from the gland the polarity as marked by the osmiophilic globules is obscured and there is a uniform distribution of the blackened material throughout the cell (Fig. 2*C*). The germinal vesicle is still intact at this stage, and no signs of the beginning of the maturation process have appeared. In this respect the behavior of the dark globules differs strikingly from that observed in *Lymnaea,* where universal and even distribution is not

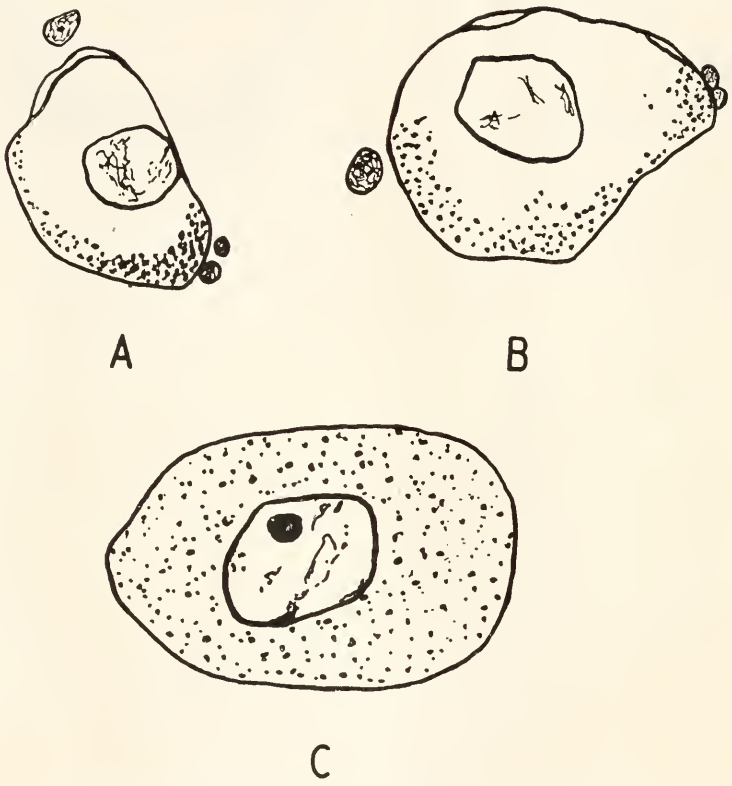

FIGURE 2. Sketches based on camera lucida tracings to show distribution of osmiophilic material during the course of development of the oöcyte of *Physa*. *A*, ovarian oöcyte; *B*, ovarian oöcyte about ⅔ developed. Compare with Figure 1*C*. *C*, oöcyte at the time of release from its envelope. Note the uniform distribution of osmiophilic material, and the fact that the germinal vesicle is still intact.

achieved until after the breakdown of the germinal vesicle. Thus the underlying system in *Lymnaea* appears more "rigid" in its composition, and though the evidence is indirect, one may speculate on the possibility that the ground substance in the oöcyte of *Physa* at the time of its release is in a more fluid physical state than that of *Lymnaea*.

No other obvious differences between species were noted, nor could other indications of the nature of the underlying cytoplasmic patterns be found. In an

effort to throw more light in this direction, living snails containing oöcytes were subjected to centrifugal force so as to displace the visible inclusions of the oöcytes and so further test their organization.

Single snails were placed on non-absorbent cotton in individual centrifuge tubes; the tubes were filled with water; and the snails centrifuged at approximately 1400 × G for thirty minutes. Care was always taken to select snails which were laying prolifically at the time of centrifugation. Of the thirty-six individuals of each species which were centrifuged, twelve were isolated in individual small-sized bowls and retained for subsequent study. The twenty-four remaining specimens were divided into six lots of four each, one of which was sacrificed immediately after centrifugation, and the remainder at intervals of ½ hour, 1 hour, 6 hours, 12 hours, and 24 hours respectively, after centrifugation. Upon excision, the gonads were fixed immediately in Champy's fluid. They were subsequently embedded in paraffin, sectioned, and stained after the Champy-Kull method. The oöcytes in these preparations were then compared with each other and with those of the other lots.

Lymnaea

In the sections of gonads fixed immediately after centrifugation, the contents of the oöcytes are stratified into three zones; one of which is centrifugal and red (yolky), one of which is almost equatorial, relatively clear and faintly pink or

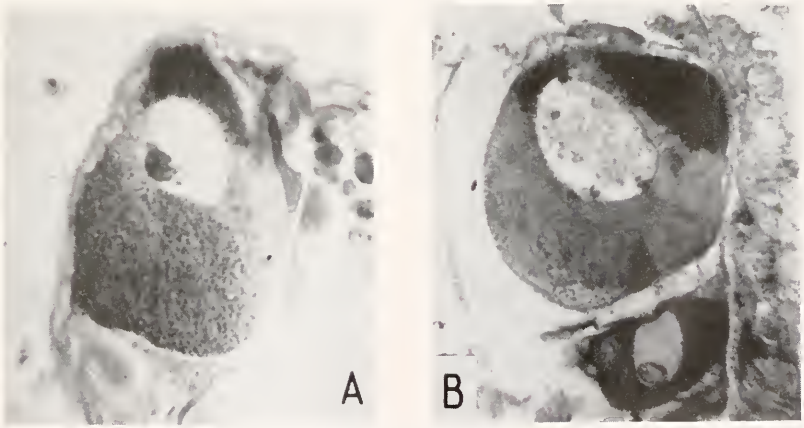

Plate II. Photomicrographs of oöcytes fixed immediately after centrifugation. *A, Lymnaea; B, Physa* (× approximately 450)

colorless (cytoplasm, or hyaloplasm), and one of which is centripetal and black (oil, blackened by osmic acid, and undoubtedly the great mass of the "osmiophilic material" referred to in the previous section). These three zones correspond, no doubt, to those described by Conklin (1910) in eggs of *Lymnaea* and *Physa* which were centrifuged after fertilization and deposition.

The nucleus in all cases lies in the clear or unstained zone, and, when compared with the controls, shows little indication of any displacement. The nucleoli in a great number of cells have been displaced centrifugally in the nucleus, though

this is not always the case. Not all of the yolky material within the oöcyte is massed at the centrifugal pole; some is usually found "trapped" on the centripetal side of the nucleus where it projects into the dark osmiophilic cap (Plate II*A*; Fig. 3*A*).

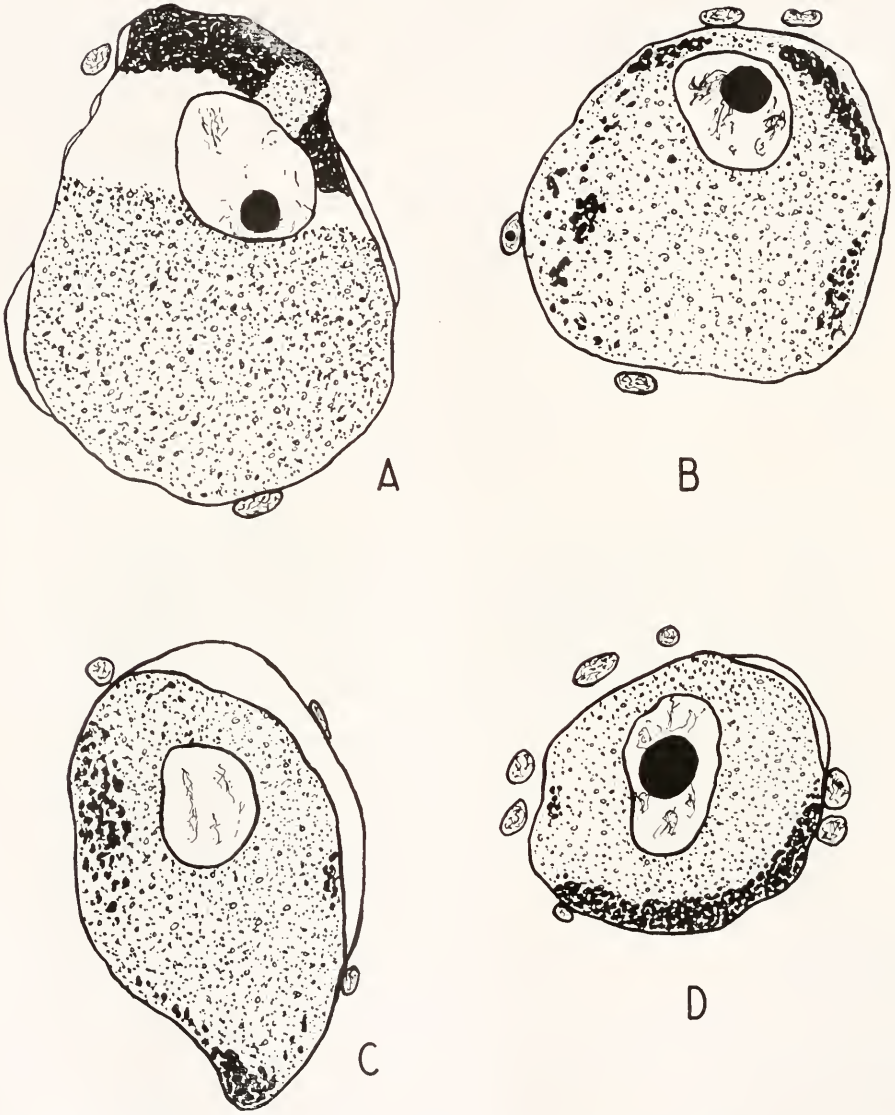

FIGURE 3. Sketches based on camera lucida tracings of oöcytes of *Lymnaea* fixed at different intervals following centrifugation. *A*, immediately after centrifugation, showing distinct layering into a black centripetal zone, clear central zone, and granular yolky zone. Granular area above germinal vesicle represents yolk "trapped" at centripetal end of cell. *B*, oöcyte fixed one-half hour following centrifugation; *C*, cell fixed one hour after centrifugation; *D*, oöcyte from preparation fixed six hours after centrifugation. Compare with Figure 1*A* and *B*.

Since the ovotestis is composed of convoluted tubules or acini, and the oöcytes are located in the walls of these acini, the orientation of the oöcytes with regard to the direction of centrifugal force varies. Stratification bears no relation to previous distribution of materials or visibly expressed polarity. This is the natural expectation based on the results of Conklin, previously mentioned.

The oöcytes in gonads fixed one-half hour after centrifugation already show a breakdown in the stratification. The "clear" hyaloplasmic area is invaded by the yolk, leaving only two of the former zones visible. The osmiophilic centripetal masses are migrating in small loose clumps from the area of former concentration, that material now being restricted to the inner cortical and subcortical regions of the cell. In the outer cortical portion between the remainder of the centripetally massed dark material and the egg surface a thin, nearly clear zone is now visible which extends over the oöcyte from the centripetal pole to a point somewhat below the middle. This zone is found one-half hour after centrifuging but is not apparent in preparations fixed immediately after centrifuging, when stratification is complete (Fig. 3*B*).

One hour after centrifugation the outer cortical clear zone has disappeared and the concentration of the osmiophilic substance has become diffused. Small clumps of the droplets are making their way back to their original position near the vegetative pole. The return path is not directly through cytoplasm and yolky materials but for the most part lies in the peripheral layers of the oöcyte. This phenomenon recalls the work of Lillie (1909) in which a denser "spongy" cytoplasmic core, evidently a part of the ground substance, was demonstrated in the unfertilized eggs of *Chaetopterus*. This center dense area is probably also in part responsible for the "trapping" of some yolky granules at the centripetal end of the oöcyte as described above.

At this stage, the yolky granules are uniformly distributed throughout the egg. The position of the nucleus has not changed, though those nucleoli which were displaced have left the centrifugal pole of the nucleus and have regained their more central location (Fig. 3*C*).

By the end of the sixth hour following centrifugation the oöcyte has, in most respects, regained its typical appearance. One or two isolated clumps of osmiophilic material may still be seen in the course of migration to the vegetative pole, but there are no other indications that the components of the cell have been displaced (Fig. 3*D*). By the twelfth hour all of the oöcytes have reassumed the appearance presented prior to stratification, so that it is impossible to distinguish the centrifuged ovotestis from that of the controls. The same is true for the gonads fixed after twenty-four hours.

Physa

The ovarian oöcytes of *Physa* fixed immediately after centrifugation show four zones. As in *Lymnaea*, there is a concentration of darkened osmiophilic substance at the centripetal pole, a clear band containing the nucleus, and a heavy red, yolky area occupying the greater part of the centrifugal hemisphere of the cell. In addition there may be found a fourth zone which is located at the extreme centrifugal pole. This appears as a small unstained area. In all probability it represents yolky or pigment material which does not react with the Champy-Kull stain,

and which does not separate out under the same centrifugal force in *Lymnaea*. Its position at the centrifugal pole would indicate that it is composed of the densest inclusions of the oöcyte (Plate II*B*; Fig. 4*A*). In general there is less interference with the migration of materials by the germinal vesicle in *Physa* than in *Lymnaea*, and when "trapping" of materials does occur, the obstructed mass is always smaller than that found in *Lymnaea*.

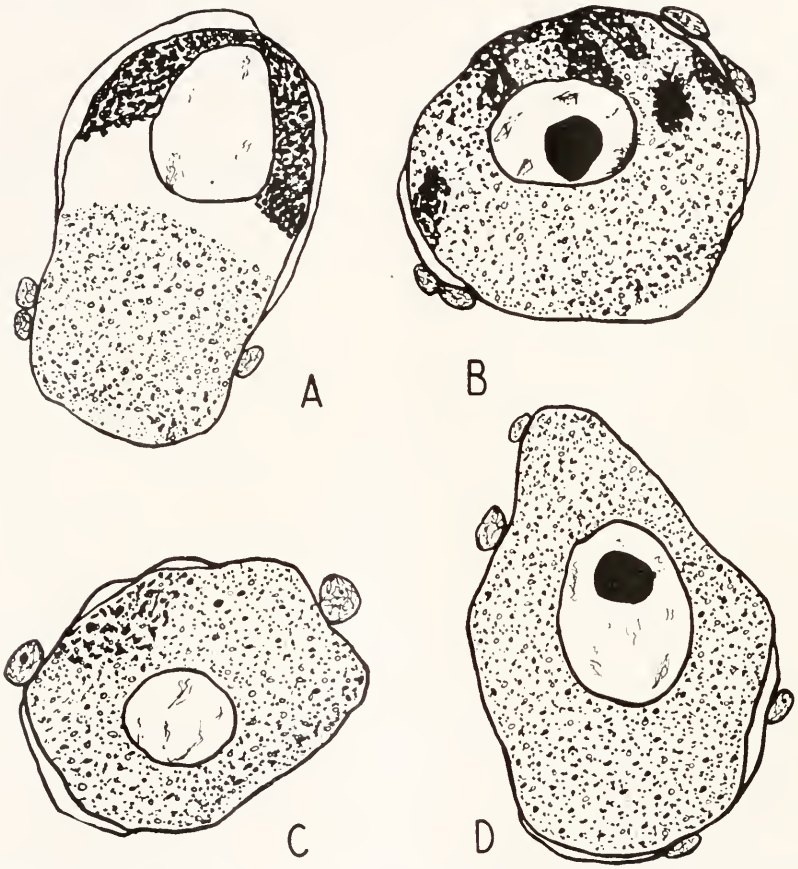

FIGURE 4. Sketches based on camera lucida tracings of oöcytes of *Physa* fixed at different intervals following centrifugation. *A*, immediately following centrifugation; the centripetal pole (black) oriented toward the head of the page, and the small centrifugal band (probably pigment) oriented toward the foot of the page. *B*, one-half hour following centrifugation; *C*, one hour following centrifugation; *D*, six hours following centrifugation. Compare with Figure 2*A* and *B*.

One-half hour after centrifugation, the materials in the oöcyte of *Physa* are fairly well redistributed. The broad, clear, hyaloplasmic band has completely disappeared; the osmiophilic concentration is rapidly breaking up into fragments which are migrating relatively freely through the cell. There is no evidence of the restricted subcortical migration noted in *Lymnaea*. The clear zone at the centrifugal pole has maintained itself, though it is, on the average, smaller (Fig. 4*B*).

One hour after centrifugation the dissociation of the clumped masses of blackened material is nearly complete. A small area is still to be found near what was the centripetal pole, but no other concentrations of osmiophilic material are visible. There is no return of the darkened substance to its previous location near the vegetative pole as in *Lymnaea* but instead these granules or droplets show a uniform distribution throughout the cytoplasm. The clear unstained mass at the centrifugal pole of the oöcyte has become definitely smaller and less distinct (Fig. 4*C*).

After six hours the oöcytes of *Physa* show no evidence of the previous stratification. The materials have been uniformly redistributed and the visible animal-vegetal differentiation which characterized the oöcytes prior to centrifugation has been eradicated. It is not regained in the oöcytes of the gonads preserved twelve and twenty-four hours after centrifugation (Fig. 4*D*).

The eggs of both species laid by the snails isolated after centrifuging were all collected, and their subsequent development was observed over a period of four weeks. In no case was any atypical cleavage or development found; nor did young snails on hatching show any differences in body symmetry from that observed in the normal animal.

On the basis of the above observations, it is possible to draw certain conclusions concerning the oöcytes of *Physa* and *Lymnaea*. We have seen first that the visible materials may be displaced without altering the developmental plan of the eggs. Second, the materials after being stratified, are redistributed rapidly and in the same sequence for each species. Hence we may postulate that some more basic organization or ground substance is present in the oöcyte, as in the deposited egg.

The variance in the manner of redistribution of materials in the two species strengthens the previous suggestion made on the basis of cytological observations of the normal oöcyte, that the physical state of this ground substance differs in the two species. That in *Lymnaea* definitely seems more fixed and not as fluid as that in *Physa*. The "trapping" of relatively large masses of yolk behind the nucleus, the clearly defined peripheral path of migration of the osmiophilic material, the exact reconstruction of the picture found prior to centrifugation, all point to this fact. *Physa* not only shows none of these phenomena characteristic of *Lymnaea* but under equal centrifugal force the materials of the oöcyte stratify into four zones instead of three, the densest inclusions separating out below the yolk in the centrifugal pole. Thus evidently, migration and separation is easier through the ground substance of *Physa* than of *Lymnaea*.

Studies With X-rays

The constancy of the developmental pattern resident in the the ground substance of the oöcyte was next tested by exposure of adult snails, and hence their oöcytes to X-rays.

The snails were placed in small, flat, glass containers and submitted to X-radiation at dosages of 200 r., 500 r., and 1000 r.[2] Fifteen individuals of each species

[2] In all runs reported, the X-rays were produced by a Coolidge type tube, having a water-cooled tungsten target. Dosage was continuous, and was administered at an approximately constant rate of 200 r. per minute.

were exposed at each value and were then isolated and observed at intervals. All egg capsules deposited after exposure to radiation were collected, placed in individual containers of pond water, and observed regularly throughout their subsequent development.

At 200 r. no effect of radiation was evident, either in the behavior of the adult snails or in the development of the eggs recovered. This was the case in both *Physa* and *Lymnaea*.

At 500 r. all of the individuals of *Lymnaea* exhibited a noticeable delay before any capsules of eggs were deposited. No eggs were recovered in any case prior to the fourth day after exposure. This was not true for *Physa* where capsules were recovered as early as twenty-four hours after the radiation of the adult snails.

In both *Physa* and *Lymnaea* the usual number of eggs in each capsule initiated development, though there was some increase in the number of eggs which did not complete their full development. The defects in development appeared usually after gastrulation, about the time of trochophore formation or immediately after. As in the 200 r. group, all snails which hatched were phenotypically similar to the parents.

At values of 1000 r. no egg capsules were recovered from either species.

The above results parallel, to a large extent, observations of Dr. E. V. Enzmann (unpublished). In discussions of his X-ray studies on *Planorbis trivolis,* he reported no success in efforts to cause monsters or reversals in this species. He was unable to find any aberration in early cleavage or in the final body plan of the adult snail, even though he was able to recover eggs from snails subjected to higher dosages than 1000 r.

It would therefore appear that the ground substance of the oöcyte, unless the cell is actually destroyed by X-radiation, is not affected adversely by it, at least in terms of the fundamental factors necessary to institute cleavage and development through gastrulation.

Discussion

A consideration of the results presented here in the light of reports by previous workers will readily bring out certain points worthy of special mention.

In the first place, the contention of Raven and Bretschneider (1942) that the visible elements of the egg are of far greater importance to ontogeny than had been previously thought, would seem to be strengthened. The shifting of the osmiophilic materials in the oöcytes of *Physa* and *Lymnaea* not only after centrifugation but also in the normal development fits in with Raven's view that inclusions shift considerably during development, and that their presence or absence in given regions of the egg or developing embryo is of primary importance. It may be pointed out again, that the shift of the osmiophilic inclusions is always the same in the oöcytes of the same species, while differing between species. This fact may well be tied in with the apparent differences in viscosity of the ground substance of the two species and is even more interesting when one considers that the cleavage pattern also differs, that of *Lymnaea* being dexitropic, while that of *Physa* is leiotropic.

In continuing a comparison of results with those of previous workers, we find some disagreement in the section dealing with centrifugation. Conklin (1910)

and his contemporaries report that following centrifugation the eggs of fresh water snails and other invertebrates stratify into three zones; and oily centripetal, a clear hyaloplasmic, and a centrifugal yolky zone. Raven and Bretschneider, in contrast, report in *Lymnaea* a fourth zone (in their work the third zone) composed of mitochondria, or "α granules," glycogen, and other inclusions, interposed between the clear zone and the centrifugal yolky zone.

The present report is in general agreement with Conklin and disagreement with Raven and Bretschneider. True, in *Physa* a fourth zone, not reported by Conklin, was found at the extreme centrifugal pole, but this is not of especial note since in these studies the oöcytes were subjected to far greater centrifugal force than was used by Conklin. Hence some additional separation of pigment or other denser inclusions from the yolk might normally be expected. However in *Lymnaea* no trace of Raven and Bretschneider's "fourth zone" between the clear and yolky bands could be found. This may be due in part to the fact that these workers in turn used somewhat higher values of centrifugal force than did the present writer. On the other hand, Raven himself reports that in certain of his preparations fixed in Champy fluid the "α granules" cannot be made out. This may rationalize the difference, since all of the preparations used in the present study were Champy fixed. Thus it is possible that the "fourth zone" was present but did not show up.

Why Champy fixation does not demonstrate this zone is an interesting cytological question. The works of Harvey (1936, 1938) using the eggs of echinoderms and of Clement (1938) using eggs of *Physa* certainly contribute good evidence in behalf of Raven's suggestion that such a zone should be largely made up of mitochondria. But a Champy-Kull stain following Champy fixation should demonstrate mitochondria. Raven and Bretschneider can offer no explanation for the discrepancy, nor is it possible for the present writer to do so on the basis of this study.

Beyond this difference the results of the sections on centrifugation and X-radiation follow closely the findings of former workers, which demonstrated that the underlying pattern of the deposited egg cannot be altered by centrifugation or similar forces. In this respect the ovarian oöcyte is evidently no different.

Summary and Conclusions

The developing oöcytes of two species of fresh water snails, *Lymnaea stagnalis* and *Physa gracilis*, are studied cytologically and experimentally through the use of X-radiation and centrifugal force. As a result of the cytological studies, certain differences in the pattern of distribution of visible inclusions are pointed out. These are specific and constant within each species, but differ between species.

The constancy of pattern within the ovarian oöcytes was tested in both species by centrifuging entire snails. Displacement and stratification of the visible cellular inclusions in the oöcytes was produced, but no aberrations in subsequent development resulted. It is concluded that a fundamental ground substance is present in the oöcyte which is not affected by the stratification of visible inclusions. The manner in which the inclusions migrated from their artificially induced stratified locations was observed, and it is postulated that the ground substance of the oöcyte of *Physa* is in a more fluid physical state than that of *Lymnaea*.

Further tests on the constancy of pattern were made by exposure of snails of each species to varying dosages of X-radiation and study of the development of

eggs laid by them subsequently. It was found that as dosage increased there was a decrease in the number of eggs which were able to complete their embryonic development. No decrease was noticed, however, in the number of eggs which were able to initiate their development. It is concluded that the cytoplasmic pattern or ground substance inherent in the oöcyte is unaffected by X-radiation of dosage values below killing intensity.

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THE RELATION OF BLOOD OXYGEN TRANSPORT TO RESISTANCE TO ANOXIA IN CHICKS AND DUCKLINGS [1]

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The relatively greater resistance of the new-born and young animals to complete anoxia in comparison with that of the adult animal has been studied by Himwich et al. (1941) who have advanced the hypothesis that differences in the aerobic and anaerobic energy release in the young and adult brains are the probable cause of the differences in resistance to anoxia. The newborn and young of many species of animals have been exposed to 100 per cent N_2, He, and CO_2 and have proved to be remarkably resistant to complete anoxia in comparison to older individuals of the same species (Fazekas et al., 1941). Since there is a total lack of oxygen in the blood stream under conditions of total anoxia and since the brain is the organ most susceptible to anoxia the oxygen requirement of the brain of newborn and young animals has been studied in comparison to the energy requirement of the brain of older animals of the same species in order to find some possible basis for the greater resistance of the young and newborn to anoxia. Himwich et al. (1941) and Chesler and Himwich (1944) have demonstrated that the anaerobic energy release in the brain of the newborn is relatively greater, in proportion to the total energy requirement of the brain, than in older individuals of the same species. After birth there is a rapid increase in energy requirement as measured by the amount of respiration carried on in the brain (Tyler and von Harreveld 1942). This increase is met, chiefly, by increase in the aerobic energy release. In the newborn the total energy requirement is low and a relatively larger proportion of the requirement is met by anaerobic energy release. The survival of the newborn and young under conditions of total anoxia is adequately explained on the basis of the relatively great anaerobic energy release in the brain of the young and newborn compared to the total energy required by the brain at this stage of development to maintain the functional integrity of the organism.

Although Quastel and Wheatley (1932) demonstrated differences in the energy requirement of surviving brain slices of cat and rat brains—the rat brain had a higher energy requirement as evidenced by greater oxygen consumption of surviving brain slices—the possibility remains that in the adult animal species differences in survival at high altitude under conditions of incomplete anoxia may be explained, in part, on several bases beside differences in brain metabolism. One of these factors is differences in oxygen transport by the blood.

[1] This work was supported by a grant from the John and Mary R. Markle Foundation to the Pathology Department. The anti-coagulant used in these experiments was "Lequaemin" supplied by Roche-Organon Inc., Nutley, New Jersey. Some of the chicks used in these experiments were given to the University of Arkansas by the Arkansas Hatchery, Little Rock, Arkansas. Research paper 595, Journal Series, University of Arkansas.

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Rostorfer and Rigdon (1945) were able to show that malarial infection greatly reduced survival time of ducks and chicks at simulated high altitude. The effect of a severe malaria infection on the survival time was marked by the third day after the birds had been inoculated with the malarial organism. At this time there was only a small percentage of red cells infected, but a shift in acid-base balance and a reduction in the oxygen carrying capacity were developing. In the study of the effect of malaria on survival of birds at high altitude a specific difference in resistance to high altitude anoxia between ducks and chicks was reported. This difference in resistance was still evident on the third day after inoculation with malaria, although there was decreased resistance in each case.

These earlier experiments suggested to the authors that specific blood differences might be related to the specific resistance to anoxia in the case of young ducks and chicks. Accordingly, a study was made of the oxygen capacities, the oxygen dissociation curves, the amounts of hemoglobin present in blood of young and adult ducks and chickens in relation to their resistance to anoxic anoxia.

Materials and Methods

White Pekin ducks and chicks of mixed breed, obtained from a commercial hatchery, between 3 and 21 days old were subjected to continuous decompression until convulsions and death occurred in a decompression chamber as previously described by Rostorfer and Rigdon (1945). Five to ten birds of the same age and species were observed during one decompression. In all experiments the rate of decompression was approximately the same with only a slight variation. The rate during the first four minutes of simulated ascent was between 1,700 and 1,800 feet per minute, between 700 and 900 feet per minute during the next 15 minutes, and 400 feet per minute during the next 40 minutes. After reaching a simulated altitude of 38,000 feet the rate of climb was such that during the next 12 minutes the altitude increased to only 40,000 feet. The altitude was recorded every 4 minutes during the decompression. These data were plotted against time and the total area under the resulting curve up to the time of death of the bird was calculated for each bird. The total area under the curve may be taken to represent the resistance of the bird to anoxia. The unit of resistance was expressed as the altitude in feet times the duration in minutes and was expressed as foot-altitude minutes.

Ducklings and chicks in groups of 7 to 12 individuals the same age were placed in the tank and decompressed until they were dead in the manner described. The ages of the various groups were 3, 6, 10, 15, and 20 days old. The average resistance to incomplete anoxia at simulated high altitude was obtained by analysis of data found by decompressing several age-groups of birds of each species. The number of birds in each separate decompression was determined by the size of the individuals.

Blood samples were obtained by heart puncture from 3 to 4 ducks or 6 to 12 chicks of the same age and pooled. Coagulation was prevented by adding 1 ml. of a 1 per cent solution of the sodium salt of heparin to each 9 ml. of blood. All red cell counts and hemoglobin and gas analyses were carried out on the pooled samples.

Oxygen capacities were determined by analysis of blood equilibrated in Barcroft tonometers with air at 38° C. Analyses were made by means of the Van Slyke manometric apparatus.

Oxygen dissociation curves were calculated for the pooled samples by equilibrating blood at a constant CO_2 tension of 31 mm. Hg and at varying oxygen tensions. Three gas mixtures were used containing 3.4, 7.44, and 11.86 per cent oxygen which corresponded to oxygen tensions of 24 ± 0.5, 53 ± 0.5, and 83 ± 0.5 mm. Hg, respectively. Analyses carried out on blood equilibrated at these three oxygen tensions gave data from which the oxygen dissociation curves were calculated by the use of the formula $y = \frac{100\ Kx^n}{1 + Kx^n}$, when the oxygen capacity of the blood was known (Hill, 1910).

Hemoglobin determinations were made on each pooled sample by the use of the Schulte and Elvehjem (1934) colorimetric method. Hemoglobin was determined, also, by calculation from the oxygen capacity. These data indicate the amount of functional hemoglobin.

Cell counts were made in the usual manner on the pooled blood and the color indices were calculated by dividing the grams of hemoglobin calculated from the oxygen capacity by the total red cell counts.

Results

In order to determine the specific resistance of young and adult ducks and chickens to the incomplete anoxia produced at simulated high altitude a series of decompression experiments were carried out in the manner described. The data represented by Figure 1 show the decreasing resistance to anoxia with increasing age. The three-day old birds of both species had the greatest resistance. There appeared to be no change in resistance between the 15- and 20-day old birds in either species. There was, however, definitely greater resistance in the duck than in the chick at all ages compared. Adult birds were not studied but there are reasons to assume that the adult duck would be more resistant to anoxia than the adult chicken.

These data indicate that the newly hatched and young birds of the same species are more resistant to incomplete anoxia produced at simulated altitude. This corresponds to the resistance of young and newborn mammals studied by Himwich and others. The data indicate, also, a specific difference in resistance to incomplete anoxia between ducks and chicks. Ducks 20 days old decompressed in the usual manner succumbed at altitudes between 34,000 and 36,000 feet, while chicks 20 days old were all dead at 27,000 feet when the decompression was at the same rate as that for the ducks.

There is little doubt that specific differences in oxygen capacity and color index exist among animals. This difference might possibly bear some relation to resistance to anoxia. The oxygen capacities and color indices were determined for all blood samples. Determination of oxygen capacities of duck and chick blood revealed that the duck blood had considerably greater oxygen capacity. Duck blood from 20-day old birds had an average oxygen capacity of 12.27 volumes per cent. Chick blood from birds the same age averaged only 10.47 volumes per cent.

The color indices of the 20-day old duck varied around 3.6×10^{-11}. Rostorfer and Rigdon (1946) found that the color indices gradually increased with age of the young ducks. The color index of chicken blood is approximately 3.1×10^{-11} and there seemed to be no increase in the color index with age as in the duck. The

color index of blood taken from the adult chicken was 3.3×10^{-11}. There was an appreciable difference in total red cell counts between the duck blood and the chick blood. The average count for the pooled blood of the chick was 2.2 million and the average count of the pooled blood of the duck was 2.6 million per cubic millimeter.

The chick blood in comparison to the duck blood had fewer red cells, decreased color index, and lower oxygen capacity.

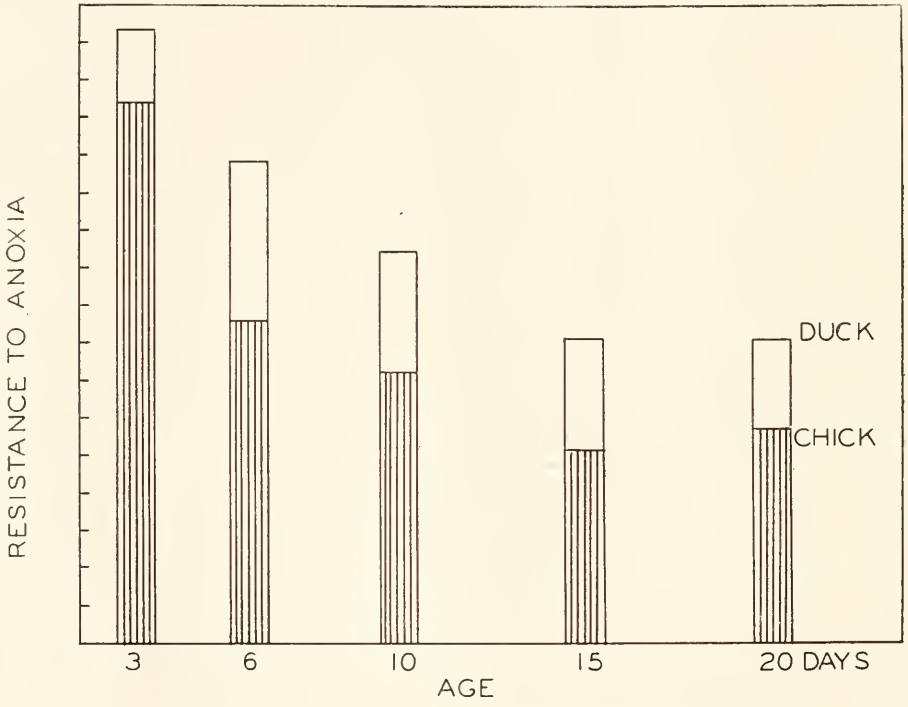

FIGURE 1 is a graphic representation of the data on the study of relative resistance of chicks and ducks to simulated altitude. Resistance is expressed in feet of altitude times the duration at altitude in minutes. Each age group represents the average data of 10 to 20 ducks or chicks.

A considerable difference between the amount of hemoglobin determined colorimetrically and that calculated from the oxygen capacity was found for young and adult ducks by Rostorfer and Rigdon (1946). A similar study was carried out on young and adult chicks. The comparison is made in Figure 2 which represents the relationship between the amount of hemoglobin determined colorimetrically and the amount of hemoglobin calculated from the oxygen capacity. The linear expression was obtained by determining the amount of hemoglobin colorimetrically and by calculation from the oxygen capacity of a diluted, a normal, and a concentrated sample of the same pooled blood. Cell counts of the three samples, dilute, normal, and concentrated, were made and the color index of each determined. There was usually close agreement between the color indices of the three samples. The young chicks and ducks carry less oxygen per gram of hemoglobin than the

adults based on the colorimetric hemoglobin. There was a slower shift toward the adult relationship with increasing age in the duck than in the chick. Thirty-day old chicks were found to have hemoglobin which corresponded to that found in the hen. The linear relation between oxygen capacity and the acid hematin of the adult chicken blood was the same as that of the young duck.

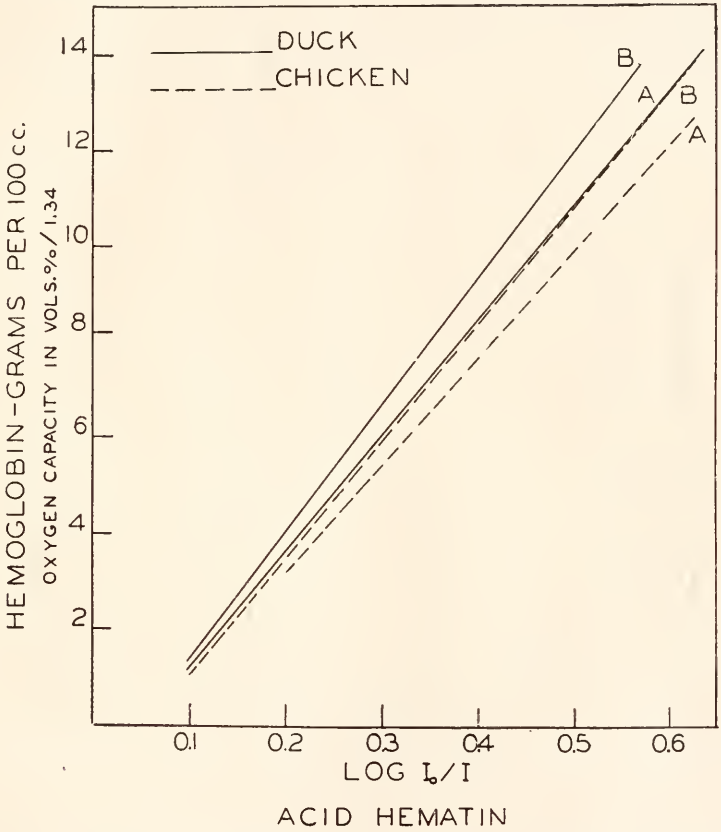

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FIGURE 2 is a graphic representation of the relationship of the amounts of hemoglobin calculated from the oxygen capacity in comparison to the amount of hemoglobin determined by photo-electric colorimetry in the blood of young (*A*) and adult (*B*) domestic ducks and chickens.

The equilibrium between hemoglobin of bird blood and oxygen at various oxygen tensions has been investigated by Wastl and Leiner (1931) whose work indicated that blood of the duck and of the pigeon does not have typical oxygen dissociation curves. Redfield (1933) points out that such curves for blood containing hemoglobin had not been described previously but were characteristic of the blood of invertebrates. Christensen and Dill (1935) were not able to confirm the work of Wastl and Leiner, but found that the blood of ducks and other birds developed equilibria between hemoglobin and oxygen in the classical manner except at very low oxygen tensions. This discrepancy they attributed to a systematic error of equilibration. Rostorfer and Rigdon (1946) found considerable difference be-

tween the dissociation curves for young and adult ducks. The blood of the adult birds gave a typical sigmoid curve while that of the young ducklings had a greater oxygen uptake at lower oxygen tensions than would be indicated by calculation from points of equilibrium at higher oxygen tensions. However, the deviation was slight.

Bloods of young and adult chickens were investigated in similar manner. Figure 3 compares the oxygen dissociation curves of adult ducks and chickens. There was a considerable difference between the oxygen uptake of the blood of the

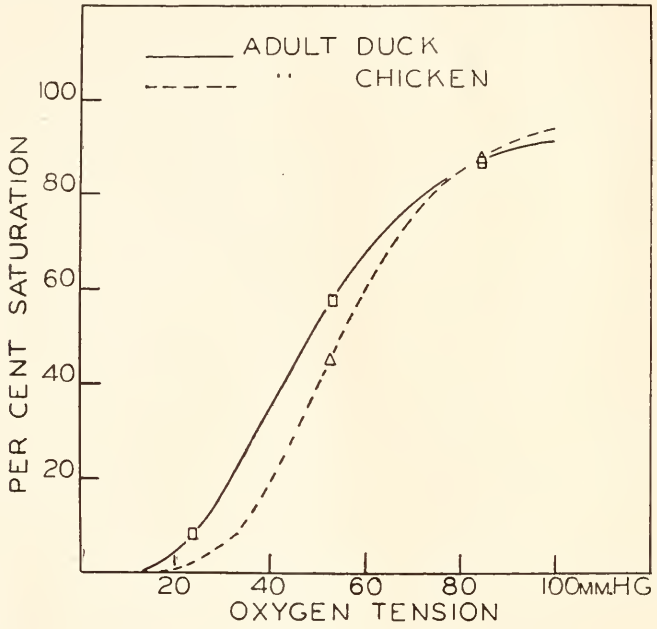

FIGURE 3 compares the oxygen dissociation curves for the adult duck and chicken at a CO_2 tension of 31 mm. Hg.

adult duck and the adult chicken at various oxygen tensions. The adult duck blood had about the same percentage of saturation at ordinary oxygen tensions but was capable of carrying a larger amount of oxygen than chick blood at low oxygen tensions.

Figure 4 compares the dissociation curves of the blood of the adult and of the young chicken. Although the curve for the blood of the young chick represents the data obtained from one sample of pooled blood only, it is reasonable to assume that it is representative. In general, the blood of the young chick appeared to have a greater percentage of saturation at all levels of oxygen tension than the blood of the adult chicken. In contrast to the blood of the adult duck, the blood of the young chick had a higher percentage of oxygen saturation at higher oxygen tensions but a slightly lower per cent of saturation at low oxygen tensions.

In general, the blood of the duck in comparison to chicken blood had greater oxygen capacity, contained more functional hemoglobin, and showed less dis-

crepancy between the colorimetric hemoglobin and the hemoglobin calculated to be present from the oxygen capacity. Duck blood had a larger number of cells and a higher color index than chicken blood. The chemical behavior of duck blood in comparison to chicken blood, as regards the oxygen dissociation curves, indicated that the duck blood would be more efficient in the transport of oxygen under conditions of incomplete anoxia. The blood of the duck in comparison to that of the chick would appear to be more capable of maintaining the brain and other vital organs in a viable condition for a longer time under conditions of simulated high altitude and resulting anoxia.

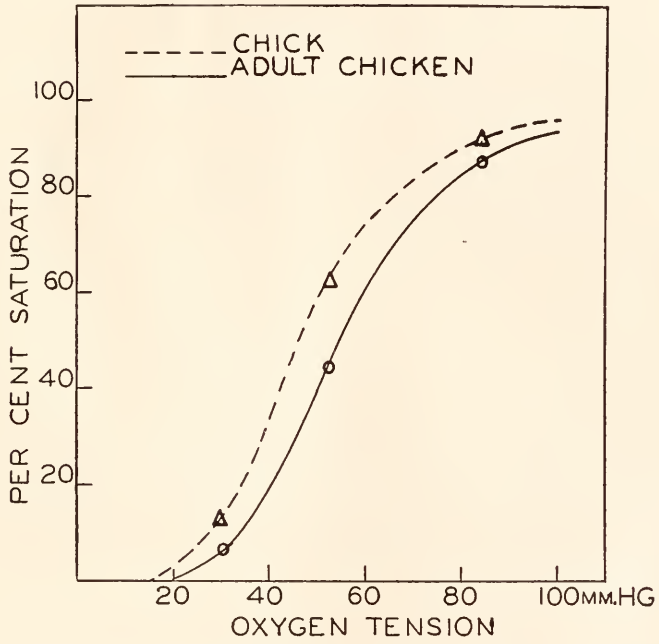

FIGURE 4 compares the oxygen dissociation curves of young and adult chicken blood at a CO_2 tension of 31 mm. Hg.

It is not unreasonable to assume that some of the difference in specific resistance to anoxia of the duck and the chick is caused by the greater efficiency of the blood of the duck at low oxygen tensions. However, a study of the anaerobic and aerobic energy release compared to the total energy requirement of the brain of the two species in question has not been made as yet. One might well assume that there are specific differences in brain metabolism.

SUMMARY

A study of the resistance of ducks and chicks to incomplete anoxia produced at simulated altitude is reported. The newly-hatched and young birds had greater resistance than older birds. The duck was found to be more resistant than the chick to incomplete anoxia at all ages studied.

A study of the oxygen carrying capacities, total red cell counts, color indices, the amount of hemoglobin, and the oxygen dissociation curves of the blood of the duck and the chick is reported.

The blood of the duck has greater oxygen capacity, higher total red cell count, higher color index, more hemoglobin and a higher percentage of saturation of the blood with oxygen at low oxygen tension when compared with blood of the chicken.

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THE FOOD-VACUOLE IN *PARAMECIUM* [1]

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INTRODUCTION

The food-vacuole has been more extensively studied in *Paramecium* than in any other organism. There is, however, still much diversity of opinion concerning the processes involved in its formation and movement and in the changes that occur in it. These phenomena will be considered in the following pages.

MATERIAL AND METHODS

Four species of *Paramecium* [2] were used in the following observations, namely, *caudatum*, *multimicronucleatum*,[3] *aurelia*, and *trichium*. All the specimens used were obtained from pure cultures maintained in the laboratory.

[1] I am indebted to Dr. W. J. Bowen for very efficient assistance in some of this work, and to the artist John S. Spurbeck, for expert service concerning the figures.

[2] Dr. D. H. Wenrich very generously identified the species used.

[3] The name *"multimicronucleatum"* is so long and unwieldy that the abbreviation, *nucleatum*, will hereafter be used in place of it.

Paramecia are usually so active that it is very difficult to study them under high magnification. Various methods have been used to quiet them, e.g., addition of narcotics (Bills, 1922), addition of yeast-cells stained with congo red (Buck, 1943), increase in viscosity (Marsland, 1943; Moment, 1944; and others). All these methods and also reduction in temperature were tried. The best results were obtained with the second method and some modifications of it.

It was found that locomotion can readily be stopped without apparent injury by means of narcotics or by increasing the viscosity of the culture fluid, but that when locomotion ceases under these conditions, feeding does also. It was also found that while movement decreases greatly with decrease in temperature, it does not decrease sufficiently to make observation under high magnification practicable until after the paramecia have been actually frozen and killed.

Nearly all the observations were made on preparations made as follows: A drop of solution containing numerous paramecia from a young, vigorous culture was mounted between two small ridges of vaseline on each of several slides. Yeast-cells stained with congo red (Buck, 1943) were added to some, chinese ink or carmine to others, and an abundance of zooglea and a little neutral red or nile blue to still others. Then all were covered with cover-glasses and sufficient fluid removed from some to flatten the paramecia considerably. All the preparations were kept in a damp chamber when not under observation.

The paramecia in these preparations usually lived for several days. They were very active at first but they soon became quiet, although it sometimes required 24 hours or more, and in some of the preparations they became so quiet that given individuals could be studied continuously for several minutes, even under an oil-immersion objective. Nearly all the quiet paramecia, even those which had been flattened, fed vigorously. In some of these, especially those which were flattened, the structure of the feeding apparatus, the activity of the cilia and the movement of particles in it, and the formation of the food-vacuoles could be clearly seen.

It was found that the paramecia from young, rapidly growing cultures become quiet much more readily than those from old declining cultures. This is doubtless, at least in part, due to difference in the hydrogen-ion concentration of the cultures. At any rate, it was observed that if a trace of alkali is added to fluid from young cultures the activity of the paramecia in it increases greatly and that if a trace of acid is added to fluid from old cultures the activity of the paramecia in it decreases.

The Feeding Apparatus

Numerous observations were made on the feeding apparatus in the four species of *Paramecium* listed above. The following conclusions were reached: The feeding apparatus is essentially the same in structure in the four species studied. It consists of a shallow ciliated groove (the oral groove) which extends from the anterior end to slightly beyond the middle of the body, a ciliated depression (the vestibulum) at the posterior end of the groove, a ciliated tube which extends from an opening in the floor of the depression (the mouth) backward into the body, and some fibers which extend from the distal end of the tube toward the posterior end of the body.

The tube is called pharynx by some, cytopharynx, gullet or esophagus by others, and a portion of it pharynx and the rest esophagus by still others. I shall call it

pharynx. The pharynx can be seen distinctly in living specimens. It extends from the mouth directly toward the center of the body a short distance, then turns backward sharply and proceeds parallel with the surface of the body for some distance, then turns sharply again but nearly at right angles to the first turn and ends almost immediately in an elliptical opening which leads into the forming food-vacuole which I shall designate "esophageal sac" (Fig. 1).

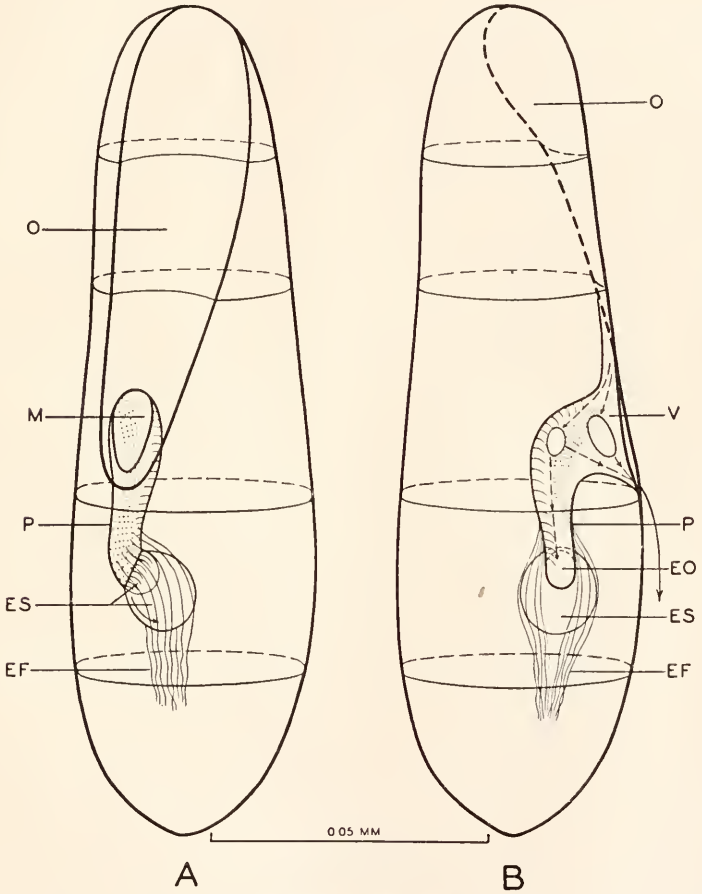

FIGURE 1. Camera outline of *Paramecium aurelia* showing the feeding apparatus and its relation to the rest of the body. *A*, oral groove and mouth facing upward; *B*, oral groove and mouth facing to the right; *O*, oral groove; *M*, mouth; *V*, vestibulum; *EO*, esophageal opening; *P*, pharynx; *ES*, esophageal sac; *EF*, esophageal fibers.

Nirenstein (1905) and others maintain that the mouth is a fixed oval opening. But Frisch (1937) says it "is a narrow slit bounded by a raised, thickened border in the form of an elongated oval, somewhat like lips" and that it is sometimes closed. His observations were made on an exconjugant individual, beginning immediately after it had separated from its mate and continuing for one and one-half hours. I have seen the mouth under the oil-immersion objective in many individuals under

various conditions but not in any immediately after conjugation. In all these, the mouth was continuously distinctly oval in outline, and I did not see any indication of change in form or size. It may well be that the phenomena observed by Frisch occur only for a few hours after conjugation.

If a paramecium is so oriented under the cover-glass that the anterior end is directed from the observer and the oral groove is to the left and faces upward, one can see down through the mouth into the proximal end of the pharynx and one can see the esophageal sac extending at right angles from the opening at the distal end. If the paramecium now rotates on its longitudinal axis 90° to the left, one can see the sharp curve in the pharynx at the proximal end and one can see down through the esophageal sac into the distal end of the pharynx. A more distinct view of this sac (the forming food-vacuole) is, however, obtained when the oral groove is to the right and faces downward, for the distal end of the pharynx is now nearer the upper surface of the body (Fig. 1).

The esophageal sac consists of a thin elastic membrane which separates the content of the sac from the cytoplasm. It changes greatly in form and size as the food-vacuole develops.

The inner surface of the pharynx contains numerous cilia. This can be seen clearly in living specimens, but no details can be made out concerning their arrangement or their size or their action, except at the distal end where a number of long cilia can be seen to beat vigorously into the forming food-vacuole.

Gelei (1934) found, in observations on fixed and stained paramecia, that there are in the pharynx two bands of cilia and that one of these bands, the "penniculus", extends from the anterior left edge of the pharynx nearly to the posterior oral edge and the other (called "Vierermembran") from the anterior aboral edge to the posterior oral edge. He maintains that the former usually contains eight rows of short cilia and the latter four rows of long cilia which at the distal end of the pharynx extend into the forming food-vacuole. Lund (1933, 1941) agrees with Gelei in reference to the composition, the location and the extent of the former and the composition and the location but not the extent of the latter. He holds that the latter does not extend to the distal end of the pharynx but that there is at this end a "pouch" which contains a "heavy tuft of cilia."

The results of my observations are in full accord with Gelei's contention. I could not see anything that resembled a pouch at the end of the pharynx, but I could see distinctly long cilia at this end, which extended into the forming food-vacuole. These however, clearly appeared to be at the end of the "Vierermembran" not in a partially separated pouch (Fig. 2).

Bozler (1924) maintains that there are attached to the right wall of the pharynx about half-way up, some ten long fibers which extend nearly to the posterior end of the body, and that these fibers are fixed in position, fairly rigid and in the same plane. He calls them "Schlundfaden" and says similar fibers occur in other protozoa and that Schuberg (1890) first discovered them in observations on *Stentor*.

Gelei (1934) and Lund (1933, 1941) confirm Bozler in reference to the presence of long fibers attached to the pharynx in *Paramecium* but they do not agree with him in the contention that the fibers are fixed and in one plane. Gelei holds that they are attached to the "right wall" of the pharynx but near the proximal end and that they are not all in one plane. Lund maintains that they are attached near the distal end of the pharynx on all sides and are not fixed. He says (1933, p. 55):

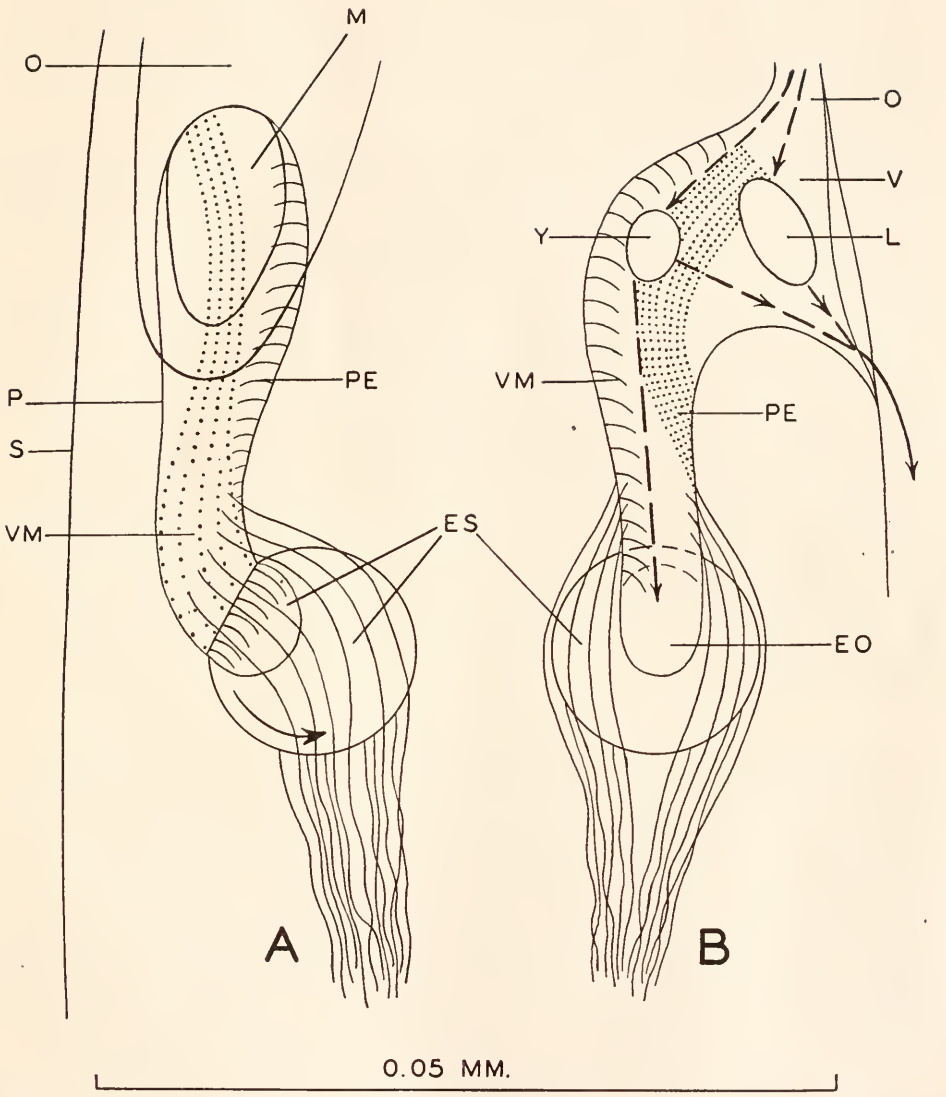

FIGURE 2. Camera outline showing the structure of the feeding apparatus in *Paramecium aurelia*. *A* and *B*, two views, one at right angles to the other. *O*, oral groove; *V*, vestibulum; *M*, mouth; *P*, pharynx; *EO*, esophageal opening; *ES*, esophageal sac; *EF*, esophageal fibers; *PE*, penniculus; *VM*, "Vierermembran"; *Y*, yeast-cell; *L*, a large particle; *S*, surface of the body; broken lines, paths taken by particles during the process of feeding; arrows, direction of movement.

The cilia which project into the esophageal sac from the end of the pharynx are doubtless part of the "Vierermembran." There are, of course, many more than are represented.

"Frequently in stained animals they are wrapped almost around the food vacuoles. In the living animal they can be observed only a short way beyond the gullet, but certainly not all in one plane and are capable of some movement. All morphological evidence points to a lax condition of these fibrils, with their distal ends free." He calls them "postesophageal fibrils".

Lund maintains that there are also "five or more heavy fibrils" which extend from their attachment at the anterior edge of the opening at the distal end of the pharynx, to the posterior edge where each ends in a "large granule" but that they can not be seen in living specimens. The larger granules, Lund thinks, are part of the neuromotor system. He calls these fibers "paraesophageal fibrils".

I have made extensive observations on numerous living specimens of the four species of *Paramecium* listed above, concerning fibers attached to the pharynx. Some of the observations were made on specimens which had been greatly compressed under the cover-glass, others on specimens stained with various vital dyes and still others on specimens in various stages of inanition. All were made with the highest grades of optical and illuminating systems (Mast and Bowen, 1944).

I could not see any fibers across the distal opening of the pharynx in any of the specimens studied, but in a few I could see several fibers which appeared to extend posteriorly from this end. I could not, however, make out any details as to their attachment or their length. This indicates that the index of refraction of these fibers is very nearly the same as that of the cytoplasm in which they are imbedded and that in this respect they differ from those found in the Peritricha for in these organisms they can readily be seen in the living state (Mast, 1944; Mast and Bowen, 1944).

The Formation of the Food-Vacuole

Paramecia ordinarily do not ingest anything when they are swimming actively (Mast and Lashley, 1916). When they are at rest or are swimming slowly in contact with a solid surface, the oral cilia produce a current down the oral groove into the vestibulum. This current contains in suspension all sorts of small solid particles many of which are carried into the vestibulum, but most of these are immediately carried out again. The rest pass through the mouth into the pharynx; some of these are thrown back out again, the rest and some fluid pass through the pharynx into the esophageal sac. Here the fluid with the particles in suspension usually rotates rapidly, and as more and more substance enters the sac, the particles in it become more concentrated and the sac becomes larger, and finally a portion of it separates from the pharynx as a full-fledged food-vacuole. All these phenomena can be seen readily and there is little disagreement concerning them; there is, however, marked diversity of opinion concerning the processes involved. These will be considered in detail under several headings in the following pages.

Selection of particles.

Metalnikow (1907, 1912) concludes that while paramecia ingest all sorts of small particles they take more of those which are digestible than of those which are not. This conclusion is supported by Bozler (1924), Losina-Losinsky (1931), Bragg (1936a, 1939), and others. It is consequently fairly certain that paramecia can differentiate between various small particles. There is, however, little known concerning the factors involved.

Bozler (1924) maintains that selection is usually made at or near the entrance to the pharynx by the response of individual cilia to contact with particles which have been carried into the vestibulum by the oral current, i.e., that if a particle comes in contact with a cilium, the cilium responds either by throwing the particle into the pharynx or into the outgoing current, depending upon the physical characters of the particle. He says (p. 189): "Man ersieht . . . dass das Hineinbefördern der Nahrungsteilchen nicht durch ein planmässiges Zusammenwirken der Cilien geschieht, sondern dass jede Cilie auf Grund der Reize reagiert, die sie direkt von dem Nahrungskörper erhält." He maintains, however, that particles which have entered the pharynx are sometimes forced back out, even after they have nearly reached the distal end, but that this occurs only if the particles are large enough to fill the lumen of the pharynx. Bozler's contention that selection in *Paramecium* is dependent upon physical properties is supported by Schaeffer (1910) in observations on *Stentor*. Nelson (1933), on the contrary, presents convincing evidence that selection, in at least some ciliates, is dependent upon chemical properties.

Losina-Losinsky (1931) holds that discrimination between particles depends upon the action of cilia controlled by a central coordinating system, rather than upon the direct response of individual cilia to contact with the particles and that the stimulating agent is chemical rather than physical. He holds that acceptance of a particle is essentially the result of a chemopositive response and rejection to that of a chemonegative response.

I made numerous observations on the movements of particles in the feeding apparatus of *Paramecium caudatum, P. nucleatum, and P. aurelia.* Most of these observations were made under the oil-immersion objective with specimens variously oriented (Fig. 1). Some of the specimens were in culture fluid which contained numerous bacteria and various other solid particles and others were in culture fluid to which chinese ink, carmine, wheat starch and yeast-cells stained with congo red had been added respectively. No difference was observed in the three species studied.

When the paramecia were actively feeding, many particles of all sorts found in the surrounding medium were carried into the vestibulum, but only a small proportion of these passed on through the mouth into the pharynx. The rest passed out again—some immediately, some not until after they had circulated around in the vestibulum for a time. Nearly all the particles which entered the pharynx were small. A few of those and all the larger ones which entered, passed rapidly on into the esophageal sac without any indication of retardation on the way. The rest immediately after they had passed through the mouth, plunged, one at a time, into the band of cilia (the "Vierermembran") on the the aboral wall of the pharynx. Here they stopped a moment and then either passed back out through the mouth and the vestibulum or directly on into the esophageal sac (Fig. 2*B*).

It consequently seems to be the cilia in the "Vierermembran" near the proximal end of the pharynx which, after momentary contact, either throw the particles back out of the pharynx or down toward the esophageal sac. I have no evidence which indicates what it is that decides the fate of these particles after they have come in contact with these cilia. I have seen many stained, dead yeast-cells of all shapes and sizes rejected after they had come in contact with them and I have seen many of the same shapes and sizes accepted. There appeared to be neither rhyme nor reason in the process under these conditions.

The results presented indicate, therefore, that selection among the larger particles takes place in the vestibulum and selection among the smaller ones in the proximal region of the pharynx and the vestibulum; but they do not illuminate the problem concerning the nature of the stimulating agent involved in the selection of either.

Some of the particles ingested were surprisingly large. Several specimens of *Chilomonas paramecium,* 18 μ long and 10 μ wide, and starch grains, 16 μ in diameter, were seen to pass through the pharynx into the esophageal sac (Fig. 2). Bozler (1924) also found that paramecia sometimes ingest extraordinarily large particles. He found starch grains 11 μ in diameter in the food-vacuoles. The ingestion of particles as large as these requires great extension of the pharynx, for it usually is, in the smallest region, only five to seven micra in diameter. This shows that the pharynx is far from being as rigid as it appears to be.

Bills (1922) and Bozler (1924) maintain that particles are often rejected after they have passed well on down the pharynx, sometimes nearly, if not quite, to the esophageal sac. They consequently imply that selection of food may take place anywhere in the pharynx. Lund (1933, 1941) seems to hold that the particles which have entered the pharynx collect in front of a sort of fibrous screen at the mouth of the esophageal sac and then gradually slip through "one by one" into it and that selection takes place here, at least in part.

I have seen hundreds of particles of all sorts pass through the pharynx into the esophageal sac under various conditions, but I have never seen one rejected after it had passed beyond the first bend in the pharynx; nor have I ever seen any indication of decrease in the rate of movement between this bend and the sac.

The enlargement of the esophageal sac and the increase in the concentration of particles in it.

When a food-vacuole forms by constriction of the esophageal sac, a portion of the sac remains as a membrane over the distal opening of the pharynx. This membrane bulges slightly out into the cytoplasm and thus forms a shallow new esophageal sac. If there is active ingestion, this shallow sac rapidly becomes deeper and finally nearly spherical in form. It contains relatively much fluid and few particles at first, but as it increases in size the concentration of particles increases (at first slowly then more and more rapidly) until the sac often appears to be almost filled with them. The long cilia which extend from the end of the pharynx into the sac beat vigorously; this causes the particles in the sac to vibrate actively and its entire content to rotate. Rotation usually continues until the sac is closed; but sometimes it ceases long before the sac is closed and then only those particles vibrate which are in close contact with the cilia at the mouth of the sac. This is doubtless due to increase in the viscosity of the fluid in the sac. The quantitative relation between fluid and solid particles in the newly formed food-vacuole varies very greatly. Under some conditions the vacuole appears to be entirely filled with fluid, no particles whatever being visible even under the highest magnification, while under others, as stated above, it appears to be almost entirely filled with particles.

Bütschli (1889) and Horning (1926) contend that the content of the pharynx is in direct contact with the cytoplasm, i.e., that no membrane intervenes at the distal opening and that the droplets (vacuoles) which pass from the pharynx into the

cytoplasm are surrounded merely by a surface film. Bütschli seems to think that this continues, but Horning holds that in the cytoplasm, membranes form at the surfaces of the droplets. Gelei (1934) concludes that the content of the pharynx is separated from the cytoplasm by a definite membrane which persists as the esophageal sac enlarges, and that this membrane is porous; for he observed in stained whole mounts, that the cytoplasm adjoining it was strongly stained, whereas that adjoining the pellicle at the surface of the body and that adjoining the wall of the pharynx was only slightly stained, if at all.

Nirenstein (1905) maintains that the cytoplasm exerts suction ("eine Art Saugwirkung") on the esophageal sac, and that this causes the sac to enlarge very rapidly to its maximum size and fill with fluid containing but few particles, and he thinks that the cilia in the pharynx then force more particles into the sac and that this causes the observed increase in their concentration. That is, he seems to hold that the cilia come in direct contact with the particles and force them through the fluid in the pharynx and finally into the sac.

Bütschli (1889), Bozler (1924) and others maintain that the enlargement of the esophageal sac is due to pressure of fluid forced into it by the action of the pharyngeal cilia. They consequently do not agree with Nirenstein in reference to the cause of the enlargement, but they appear to agree with him in reference to the process involved in the concentration of particles. Bozler describes this as follows: A drop consisting largely of water ("ein Wassertropfen") is first formed; then solid particles are forced into it by the cilia in the pharynx ("hereingestrudelt") and whirled about by those which project from the end of the pharynx into the drop. After the drop has become nearly filled with particles these cilia rotate its content and press the particles together so as to get as many in as possible.

All the investigators referred to obviously hold that when the esophageal sac begins to enlarge almost nothing but water enters, that this continues until it has nearly, if not entirely, reached maximum size and that after this almost nothing but solid particles enter. This would account for the observed increase in the concentration of solid particles in the esophageal sac, but it is difficult to understand why the cilia in the pharynx should at first transport mainly water and later mainly solid particles.

Lund (1933) agrees with Bütschli and Bozler in reference to the cause of the enlargement of the esophageal sac, but he thinks that "paraesophageal fibrils" at the mouth of the sac are involved in the concentration of particles in it. He says (1933, p. 54): "Food material collects as it reaches these fibrils, so it is probable that they too aid in the concentration of food particles into the vacuoles" and (1941, p. 564) "Here particles of all sizes are trapped in the paraesophageal fibrils, while most of the fluid material presumably circulates back into the pharyngeal cavity. One by one the particles of food slip through the paraesophageal fibrils, and arrive in a growing bulge (the future food vacuole) on the dorsal side of the pharynx."

Lund found in section of paramecia, some particles which appeared to have been entangled in fibrils at the mouth of the esophageal sac. This led to the conclusions quoted above. I have, however, as previously stated, been unable to see in living paramecia any indication of an aggregation of particles at the mouth of the sac or cessation of movement of individual particles in this region. Nor have I been able to find any indication of backward circulation of fluid in the pharynx. The

particles observed by Lund among fibrils at or near the mouth of the sac may well have been deposited there by the microtome knife in sectioning the organisms.

It seems to me to be perfectly obvious that the cilia in the pharynx force fluid with particles in suspension into the esophageal sac and that it is this that causes the enlargement of the sac as Bütschli, Bozler and others maintain. But I could not observe any change in the concentration of the particles in the fluid forced into the sac, during its enlargement, i.e., I did not obtain any evidence indicating that the concentration was at first low and later high as Bozler's view demands. What then causes the observed increase in concentration?

Discussion

Nirenstein (1905) maintains that after the food-vacuole has been formed and has left the pharynx it rapidly decreases in size and that this is due to loss of fluid. These contentions have been abundantly confirmed. The membrane around the food-vacuole is therefore pervious to water, and the loss of water, which causes the vacuole to decrease in size, is doubtless due to excessive external osmotic concentration and inward pressure of the stretched vacuolar membrane (Mast and Bowen, 1944).

If this is true, water must be continuously forced out of the esophageal sac during its enlargement, i.e., during the formation of the food-vacuole, for the factors involved in the loss of water from the fully formed food-vacuole function continuously during its formation. And since no solid particles leave during this time, it is obvious that this would result in increase in their concentration in the forming vacuole. But it does not account for the apparent observed increase in the rate of their concentration as the sac enlarges.

The difference between internal and external osmotic concentration is doubtless practically constant during the enlargement of the esophageal sac, as is also the pressure of the fluid against its inner surface, caused by the action of the cilia in the pharynx. The rate of outflow of water *per unit area* of membrane at the surface of the sac (provided there is no change in its permeability) is therefore practically constant; but since this area increases as the sac increases in size the rate at which water leaves the sac, i.e., the volume per second, also increases. Therefore, since as the sac increases in size the rate of inflow of fluid and particles through the pharynx remains practically constant, while the rate of outflow of fluid without particles increases, the rate of concentration of particles in the fluid in the esophageal sac must increase as the sac enlarges. Moreover, as the esophageal sac enlarges the membrane at its surface is continuously stretched, and as it is stretched it must be continuously built up by the interaction between substances in the sac and the adjoining cytoplasm so as to prevent rupture. It may well be, however, that the membrane thus formed becomes more and more pervious to water as it is stretched during the enlargement of the sac. If this is true, it also causes an increase in the rate of concentration of particles in the sac.

The conclusion that water continuously passes from the esophageal sac into the cytoplasm has an important bearing on various views concerning the origin of the water which is excreted by the contractile vacuoles. Eisenberg (1925) and Frisch (1937) hold that all this water enters the body through the pharynx; Bozler (1924), Fortner (1926), and Müller (1932) conclude that some of it enters through the

pellicle, and Kitching (1934, 1936, 1938) assumes, in some of his experiments, that all enters through the pellicle.

It has been demonstrated by several investigators that the total volume of the food-vacuoles (at maximum size) formed in a given period of time is only about one-third as great as the total volume of the contractile vacuoles (at maximum size) formed during the same length of time. This seems to prove that water enters the body through the pellicle as well as through the pharynx. Eisenberg, Frisch and others maintain, however, that some of the water that enters the pharynx passes directly into the cytoplasm, i.e., without entering the food-vacuole at all, and that this accounts for the observed difference between the total volume of the food-vacuoles and that of the contractile vacuoles. The evidence presented above that water continuously passes from the esophageal sac into the cytoplasm during the formation of the food-vacuoles, strongly supports the contention that the water excreted by the contractile vacuoles enters the pharynx; for the formation of the food-vacuole requires sufficient time to permit, during its formation, the passage of more water from it into the cytoplasm than passes from the food-vacuole into the cytoplasm after the vacuole has been fully formed.

In view of these and other considerations it is difficult to understand how Kitching, in the experiments referred to above, came to assume that all the water that was excreted by the contractile vacuole entered the body through the pellicle.

The Separation of the Food-Vacuole from the Pharnyx and its Movement through the Body

There has been for many years much speculation concerning the processes involved in the separation of the food-vacuole from the pharynx and its movements after it has become free, but these problems still remain unsolved.

I have closely observed under the oil-immersion objective, numerous food-vacuoles leave the pharynx and pass through the body in each of the four species of *Paramecium* listed above, especially the first three. The specimens studied were under many different environmental conditions, both natural and artificial. Extensive variations were seen in each species but the results as a whole seem to show that the processes involved in the closing of the esophageal sac, the separation from the pharynx of the food-vacuole thus formed, and its passage through the body are fundamentally identical in the four species.

The following usually occurs: The esophageal sac enlarges and becomes nearly spherical with the major axis directed backward. The substance in the sac rotates strongly and the particles in suspension vibrate vigorously and become much concentrated. After the sac has become about twice as wide as the pharynx, it slides posteriorly from the diagonal end of the pharynx and becomes definitely pear-shaped, a nipple being drawn out on the vacuole as it leaves the pharynx. (No change whatever in the size of the end of the pharynx can be seen during this process.) A small portion of the esophageal sac remains as a membrane over the opening in the pharynx. This bulges out into the cytoplasm slightly, forming a shallow sac which soon enlarges to form a new vacuole (Fig. 3). After the nipple which connects the newly formed food-vacuole with the tip of the diagonal end of the pharynx has broken, the vacuole moves rapidly *through* the cytoplasm nearly to the posterior end of the body, and on the way it creates marked currents in the

cytoplasm, turns through approximately 270° and becomes spherical. After it has reached the end of this course, it stops a few moments, then slowly passes forward near the aboral surface to the anterior end of the body or part way, and then back along the opposite surface, to the anus.

As stated above, there is great variation in these phenomena. Some of these are set forth in the following paragraph:

During the separation of the food-vacuole from the pharynx, the cytoplasm around it ordinarily flows slowly backward and appears to elongate the vacuole slightly, but sometimes this cytoplasm does not flow at all and occasionally it flows so violently that it appears to elongate the vacuole greatly and actually pull it from

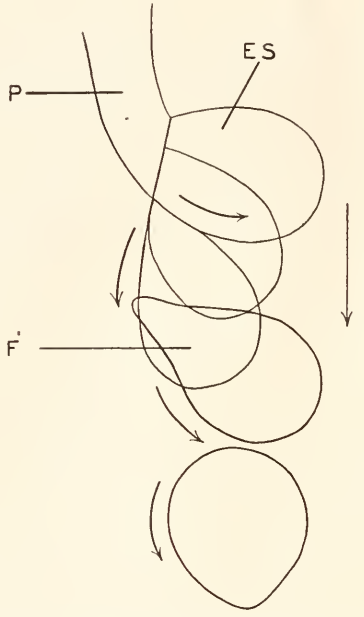

FIGURE 3. Camera outline of a portion of the feeding apparatus and some sketches illustrating the movement of the food-vacuole during its separation from the pharynx in *Paramecium aurelia*. *P*, pharynx; *ES*, esophageal sac; *F*, food-vacuole; arrows, direction of movement.

the pharynx. After the vacuole has been separated from the pharynx it almost invariably moves toward the posterior end of the body much faster than the surrounding cytoplasm, but occasionally it moves so slowly that it appears to be carried by the cytoplasm. On its way toward the posterior end of the body the vacuole usually turns somewhat more than half-way around but sometimes it does not turn at all, and sometimes it turns entirely around and occasionally even more than once; and it sometimes goes only a short distance on its usual course toward the posterior end of the body and is then carried forward. The vacuole usually becomes spherical before it has reached the posterior end of the body, but it sometimes does not become spherical until much later, if at all. This appears to be closely correlated with the viscosity of its content. Details concerning these phenomena are given in the next section (see also page 55–57).

Discussion

It is held by all who have investigated the subject that after the food-vacuole has reached the posterior end of the body, it is carried over the rest of its course in the cytoplasmic stream, i.e., by cyclosis, but there is marked diversity of opinion concerning the processes involved in the separation of the food-vacuole from the pharynx and its rapid movement toward the posterior end of the body.

Stein (1859), Bütschli (1889) and Bragg (1935, 1936) conclude that contraction of the distal end of the pharynx and cyclosis are involved. However, since no decrease in the size of the pharynx can be seen and the vacuole sometimes leaves the pharynx and moves away in the total absence of cyclosis, neither of these two factors is essential.

Nirenstein (1905) maintains that the cytoplasm adjoining the esophageal sac near the distal end of the pharynx contracts and separates the content of the sac from that in the pharynx and that the vacuole thus formed is carried away by cyclosis. Bozler (1924) supports Nirenstein in reference to the separation of the vacuoles from the pharynx, but he holds that the vacuole is not carried off by cyclosis. He contends that there is periodic backward streaming of the cytoplasm (which is not cyclosis) adjoining a group of fibers ("Schlundfaden") which extend from the pharynx nearly to the posterior end of the body and that the vacuole is carried posteriorly in this stream.

If the constriction in the esophageal sac is caused by the adjoining cytoplasm, one would expect to find a differentiated structure in the cytoplasm, i.e., a sort of sphincter. Nothing of this sort has, however, been found; and Bozler's contention that there is a stream of cytoplasm backward along one side of a bundle of fibers has not been confirmed. It is consequently not likely that the formation and movement of the vacuole is dependent upon either of these two postulated factors.

Kalmus (1931) and others seem to think that surface tension plays a predominant role in the formation of food-vacuoles, somewhat as it does in the formation of drops of water in the air. The formation of drops of water in air by the action of surface tension is, however, dependent upon weight. But since food-vacuoles suspended in cytoplasm have no weight, surface tension cannot be essential in their formation.

Gelei (1934) says the food-vacuoles are torn from the pharynx, but he does not say by what means.

Lund (1941) concludes that the food-vacuole is separated from the pharynx and forced toward the posterior end of the body by the action of the "postesophageal fibrils". He says (p. 564): "With the growth of the vacuole the postesophageal fibrils contract about its base, the vacuole is pressed posteriorly, and once it is released the fibrils conduct it into the cytoplasm with considerable rapidity. Their concerted action produces an effect somewhat resembling that produced by a peristaltic wave in the esophagus of higher vertebrates."

Lund did not actually see the processes described nor could I see anything of the sort. I believe, however, that his postulations are eminently sound, for they reasonably account for nearly all that has been seen. There is, however, one observation that seems to require further elucidation.

Nirenstein (1905, p. 451) maintains that if the food-vacuole is directly over or under the diagonal opening of the pharynx which now appears as a clear oval

("heller Oval"), it can be seen that as the vacuole leaves the pharynx, the minor axis of the oval decreases until the oval becomes a slit ("ein heller Spalt") before the major axis decreases. I could not see these phenomena, but the observations are so difficult that this casts no reflections on their validity. Nirenstein thinks that the decrease in the minor axis, before the major axis decreases, must be due to contraction of the cytoplasm ("Plasma") adjoining the esophageal sac near its connection with the pharynx. Lund asserts, as previously stated, that there are several fibers which extend over the opening at the distal end of the pharynx, i.e., the mouth of the esophageal sac. I have already presented evidence which shows that if these fibers exist (and Lund's figures seem to prove that they do), they are not inside of the pharynx or the sac. They must, therefore, be outside, some doubtless being on either side of the sac close to the end of the pharynx. If this is true, these two groups of fibers become much separated in the middle and stretched as the sac enlarges. And if they now contract the opening of the sac will be laterally squeezed and consequently will become slit-like, i.e., it will assume a form which is in accord with Nirenstein's contention. It may well be, therefore, that these fibers (called "paraesophageal fibrils" by Lund) function in the separation of the food-vacuole from the pharynx.

Initiation of the Separation of the Food-Vacuole from the Pharynx

The earlier investigators held that in *Paramecium* the size of the food-vacuole controls the initiation of its separation from the pharynx, and this idea seems still to be widely held. For example, Bragg (1935) says the food-vacuole "grows gradually larger till, reaching its full size, it suddenly drops into the endoplasm." This contention is supported by the fact that in a given individual successive vacuoles are usually nearly the same in size, but it does not account for the great difference in their size often observed.

Bozler (1924) asserts that the separation of the food-vacuole from the pharynx is initiated by contact of particles with the inner surface of the vacuolar membrane. He bases this assertion on his contention that paramecia will not form food-vacuoles in a medium which does not contain particles. In this contention he does not, however, agree with Schewiakoff (1894), who maintains that particles are not necessary. Bragg (1935) says that in *Paramecium trichium* contact of large particles with the inner surface of the vacuolar membrane is always immediately followed by separation of the vacuole from the pharynx. This seems to support Bozler's contention. Bragg says however that many of the food-vacuoles formed in this species have no large particles and that in *Paramecium caudatum* large particles in the vacuole have no effect on their separation from the pharynx. He consequently concludes that they are not necessary.

I made many observations concerning this on each of the four species of *Paramecium* under consideration and found in all these species that the food-vacuole frequently leaves the pharynx immediately after a large particle has entered but that this does not always occur in any of them. I observed that when *Paramecium trichium* is ingesting yeast-cells, the food-vacuole usually leaves the pharynx immediately after one of these cells has entered but that occasionally it does not leave until after two have entered. I also found that this obtains for each of the three other species studied, when they are ingesting specimens of *Chilomonas paramecium,* and in these

I observed that occasionally the vacuole did not leave the pharynx until after three had entered.

These results are therefore not in full accord with Bragg's contentions. However, I also observed in all the species, food-vacuoles which had no large particles whatever. This supports Bragg's conclusion that contact of large particles with the vacuolar membrane is not necessary for the separation of the vacuole from the pharynx. This conclusion is also supported by the fact that I have under various conditions seen many food-vacuoles form in which no particles whatever could be seen. It is therefore very probable that in Bozler's observations the failure of the paramecia to form food-vacuoles in a particle-free medium was due to injurious action of the medium he used rather than to the absence of particles in it.

The evidence in hand obviously throws but little light on the processes involved in the initiation of the separation of the food-vacuole from the pharynx in paramecia, and this is also true for other forms. Mast and Bowen (1944) considered this problem theoretically in reference to the Peritricha and reached the following conclusions: "It is highly probable that waves start at fairly definite intervals in the pharyngeal ring and pass simultaneously down all the esophageal fibers and that each of these sets of waves initiates a constriction in the esophageal sac, if it contains sufficient fluid to make a constriction possible. If this is true, the size of the vacuole is correlated with the rate at which the fluid is forced into the esophageal sac by the cilia in the pharynx and the rate at which it leaves this sac by osmosis. If these processes and the interval between successive waves depend upon the composition of the surrounding fluid, the temperature and the physiological state of the organism, it accounts for the observed variation in the size of the food-vacuoles and the intervals between their formation." It seems to me that these conclusions apply equally well to *Paramecium*. Moreover, Bozler (1924) says that in this genus a momentary current passes posteriorly along the "Schlundfaden" at about 50-second intervals. These currents, he asserts, are entirely independent of cyclosis. I have seen similar currents in this region, though not very distinctly. I believe they are produced by periodic action of the esophageal fibers. If this is true, it supports the conclusions presented above.

Size, Rate of Formation, and Shape of the Food-Vacuole

Size

It is well known that the food-vacuoles formed in *Paramecium* vary greatly in size, but very little is known concerning the cause of this variation.

Metalnikow (1912) maintains that if paramecia are transferred from a solution which is poor to one which is rich in digestible substance (i.e., bacteria, milk, egg-yolk, etc.) the first vacuole formed is always huge, judging from his figures, nearly 40 times larger than normal vacuoles. He also maintains that in solutions which contain only indigestible particles (carmine, chinese ink, etc.) the food-vacuoles formed are abnormally small. He does not designate the species studied, but his figures indicate that it probably was *aurelia*.

I repeated Metalnikow's experiments and obtained results which on the whole support his contention; but I found that the size of the vacuoles is not at all closely correlated with the composition of the surrounding medium. That is, I found that

the extent of change in the size of the vacuoles formed after transfer to a given medium varied greatly.

Dogiel et al. (1927) made observations on the food-vacuoles formed by paramecia [4] in solutions of salts, containing numerous particles of chinese ink in suspension. They found that in solutions of $MgCl_2$, $MgSO_4$, and $FeSO_4$, the food-vacuoles formed are long, tubular and coiled ("Nahrungsschlauche") but that in solutions of $BaCl_2$ they are minute and spindle-shaped. Judging from the sketches presented, the former would average about 60 μ in diameter in spherical form, and the latter only about 4 μ. Both were filled with particles of ink.

I repeated these experiments on each of the four species of *Paramecium* listed above, with each of four brands of chinese ink in a much greater range of concentrations of the salts than was used by Dogiel et al. Moreover, the solutions of all the salts were made up respectively in distilled water, tap-water, and fluid taken from the cultures. The paramecia ingested the particles of ink freely in all the salt concentrations tested, except those which were so high that they were injurious; but I observed no indication of the formation of abnormal food-vacuoles in any of the solutions, either in form or in size.

Dogiel et al. assume that the abnormal food-vacuoles they observed were due to the action of the salts used. They say, however, that they did not obtain such vacuoles if the chinese ink was omitted. This, and the fact that I obtained no abnormal vacuoles, strongly indicates that the abnormality observed by them was due to the action of the ink they used rather than the salts.

Cosmovici (1931) maintains that *"Colpidium colpoda"* in culture fluid containing "amylodextrine" forms tubular food-vacuoles, some of which extend from the pharynx to the anus. He concludes that there is in the ciliates a very complicated closed capillary digestive system, through which substance is moved by waves of cytoplasmic contraction, and that cyclosis is an optical illusion, due to this movement. Amazing conclusions!

I repeated and extended Cosmovici's observations using paramecia in place of colpidia, but obtained no evidence whatever in support of his contentions.

Frisch (1937) made very extensive measurements on food-vacuoles in specimens of *Paramecium nucleatum* taken from given cultures on successive days for three weeks or more. The cultures remained in a flourishing condition for nearly two weeks and then declined. During the flourishing period the average diameter of the food-vacuoles formed in different individuals varied from 17.25 μ to 25 μ with 65.55 μ for the largest vacuole measured. As the cultures declined the food-vacuoles formed decreased in diameter "first to 13.80 microns, then progressively to 10.35, 6.90 and 3.45 microns." The paramecia were plump and ranged from 227 μ to 330 μ in length until the cultures began to decline, then they gradually became thin and somewhat shorter. It is consequently evident that in these cultures the size of the food-vacuoles formed varied directly with the size of the paramecia. Frisch maintains, however, that in well-fed paramecia the size of the food-vacuoles is not closely, if at all, correlated with the size of the body and that the observed decrease in the size of the food-vacuoles during the decline of the cultures was largely if not entirely, due to decrease in quantity and quality of food, i.e., bacteria.

[4] The species is not designated but the size of the paramecia calculated from the sketches presented and the fact that only one micronucleus is figured, indicate that it was *caudatum*.

I repeated the observations made by Frisch and extended them to other species. I also made observations on the effect of increase in the viscosity of the surrounding fluid. The results obtained are in full accord with those obtained by Frisch. I did find, however, that while within a given species, the size of the food-vacuoles does not appear to be correlated with the size of the individuals, the vacuoles formed by the two smaller species (*aurelia* and *trichium*) were, in general, much smaller than those formed by the two larger (*caudatum* and *nucleatum*) and that the size in all the species appears to depend upon the viscosity of the surrounding medium.

In the observations on the effect of viscosity, polyvinyl alcohol or methyl cellulose was added to culture fluid containing paramecia on a slide. It was found that if the viscosity of the fluid became high enough to retard locomotion, but not high enough to inhibit it, the paramecia ingested the fluid rapidly, formed unusually large food-vacuoles and soon became well filled with them (Fig. 4). In some of these vacuoles a considerable number of solid particles could be seen but in others none

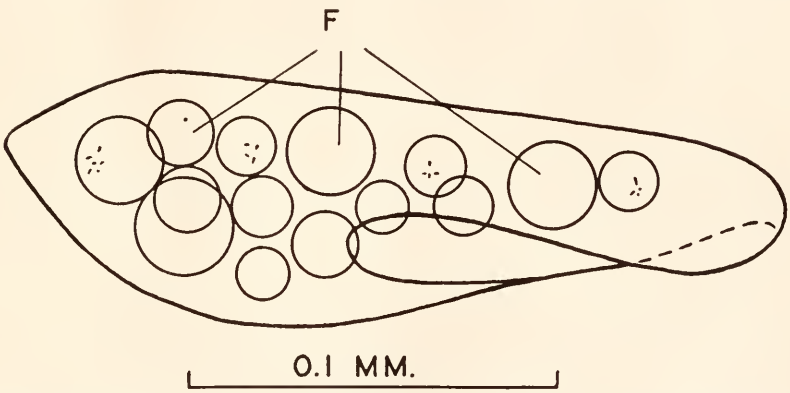

FIGURE 4. Camera outline of *Paramecium caudatum* showing food-vacuoles formed in a viscous solution of polyvinyl alcohol. *F*, food-vacuoles. The small dots in the food-vacuoles represent solid particles. Most of the vacuoles did not contain any.

whatever, despite thorough examination of much flattened specimens under the oil-immersion objective. Bozler (1924) obtained somewhat similar results with other viscous substances.

Lee (1942) asserts that "preliminary observations" show that the size of the food-vacuoles in *Paramecium* is independent of the hydrogen-ion concentration of the surrounding medium, but he gives no details regarding the results of the observations made. It may well be, therefore, that this assertion is not strictly true. However this may be, the evidence in hand seems to show that there are at least four environmental factors which are involved in the control of the size of the food-vacuoles, namely, the quantity and the quality of the particles in suspension, and the chemical composition and the viscosity of the surrounding fluid. The question now arises as to how these factors function.

Mast and Bowen (1944) found in observations on *Vorticella similis* that the food-vacuoles vary *greatly* in size without any variation in the number or kind of particles suspended in the surrounding fluid or in its chemical composition. But

they did find that under certain conditions the size is definitely correlated with the quantitative rate of ingestion.

They postulate, as stated above, that the food-vacuole is formed by constriction in the esophageal sac caused by the action of the esophageal fibers and that this constrictive action occurs at fairly regular intervals regardless of the size of the esophageal sac. If this is true, it is obvious that if the constrictive action occurs when the sac is large, a large vacuole will be formed and that if it occurs when the sac is small, a small vacuole will be formed; and it is also obvious that if the quantitative rate of ingestion is high the esophageal sac will become, during the interval between successive constrictive actions, larger than if it is low. But the size of the sac will also depend upon the quantitative rate of passage of fluid from the sac through its surface membrane into the adjoining cytoplasm. According to this view then the primary factors involved in controlling the size of the food-vacuoles are (1) the quantitative rate of ingestion of fluid and solid particles, (2) the quantitative rate of passage of fluid from the esophageal sac into the cytoplasm, and (3) the length of the intervals between consecutive constrictive actions of the esophageal fibers. If this obtains all other factors that may be involved (the quantity and the quality of particles in the surrounding fluid, the chemical composition of this fluid, the physiological state of the organisms, etc.) function by modifying the primary factors.

This hypothesis is in full accord with the facts in hand, concerning the control of the size of the food-vacuoles in the Peritricha, and it also appears to account for all that is known concerning this phenomenon in *Paramecium*.

Rate of formation

Metalnikow (1912) contends that the rate of formation of food-vacuoles is dependent upon the hydrogen-ion concentration and upon the temperature of the surrounding fluid but that it is not dependent upon the number of particles suspended in it.

Bozler (1924) asserts that the formation of a food-vacuole requires 4 to 5 minutes if the particles in suspension are scarce and only 1 to 2 minutes if they are abundant. He consequently does not agree with Metalnikow in this respect.

Frisch (1937) seems to agree with Bozler in reference to the correlation between the concentration of particles and the rate of formation of food-vacuoles. He found that the time required for the formation of the food-vacuoles measured in the observations described above, varied from 17 to 365 seconds, and he maintains that the time required for the formation of a food-vacuole is not dependent upon the size of the vacuole but that it is largely, if not entirely, dependent upon the quantity and the quality of the food present. He writes: "This variation did not depend upon the size of the food-vacuole formed but probably upon the quantity and the quality of bacteria present in the immediate vicinity of the animals." He does not elucidate this dependence, but he doubtless holds that the time varies inversely with the quantity of bacteria and the extent of their usefulness as food.

Lee (1942) demonstrated that the rate of formation of food-vacuoles is closely correlated with the hydrogen-ion concentration and the temperature of the surrounding fluid. He consequently supports Metalnikow.

No definite views have been expressed as to how the factors involved act in the

control of the rate of formation of food-vacuoles. Lee implies, however, that the rate is directly proportional to the activity of the cilia in the vestibulum ("peristome") and that this is correlated with acidity, temperature, etc. This would account for the facts observed by Lee, but it would not account for the correlation described above between the rate of formation of vacuoles and the concentration of particles in suspension in the surrounding medium. Moreover, I have repeatedly seen paramecia in which the cilia in the vestibulum were very active and numerous particles entered the vestibulum, but none passed into the pharynx, all being thrown out. This indicates that the amount of substance which enters the pharynx depends upon the nature of the activity of the cilia in the vestibulum quite as much as upon the magnitude of the activity.

If the food-vacuoles are separated from the pharynx by constrictive actions of the esophageal fibers and these actions occur at regular intervals in accord with the hypothesis presented above, the rate of formation of the vacuoles must depend upon the length of these intervals; and the effect on the rate of formation produced by the factors considered above, and others (i.e., acidity, temperature, physiological states, etc.) must be due to alteration produced by them, in the length of the intervals.

Shape

It is generally agreed that the newly formed food-vacuoles in *Paramecium* are usually nearly spherical but that they sometimes are distinctly spindle-shaped (Nirenstein, 1905; Bozler, 1924; Dunihue, 1931; Gelei, 1934; Bragg, 1935, 1936). There is no controversy concerning the cause of the spherical form, but there are several views as to the cause of the spindle-shape.

Nirenstein holds that the spindle-shape is due to constriction of the distal end of the forming vacuole by contraction of the adjoining cytoplasm and to drawing out of the proximal end to a point as the vacuole leaves the pharynx. Bozler maintains that the food-vacuole is pressed against a bundle of fibers ("Schlundfaden") on its way from the pharynx to the posterior end of the body and that this causes the spindle-shape. Gelei found that the portion of the esophageal sac which remains attached to the pharynx after a food-vacuole has left, is sometimes pointed and he maintains that this results in a point on the distal end of the following vacuole and consequently in a spindle-shape. Mast and Bowen (1944) found that this regularly occurs in the Peritricha in which the newly formed food-vacuole is always spindle-shaped. Their results consequently support Gelei's contention. Bragg (1936) concludes that the food-vacuole, immediately after it leaves the pharynx, is usually spindle-shaped in *Paramecium caudatum,* rarely in *P. nucleatum* and never in *P. aurelia* and *P. trichium.* He concludes that its form is a species characteristic, but he gives no information as to why, in a given species, it is sometimes nearly spherical and sometimes definitely spindle-shaped.

I found in observations on paramecia which had been in culture fluid containing neutral red in moderate concentration, that nearly all the food-vacuoles formed in *P. caudatum* and *P. nucleatum,* but none of those formed in *P. aurelia* and *P. trichium,* were spindle-shaped. I also found that, while in the spherical vacuoles there was invariably violent movement, in the spindle-shaped vacuoles there was little or none. This shows that the viscosity of the fluid in the vacuoles differs greatly and that the shape of the vacuoles is correlated with it.

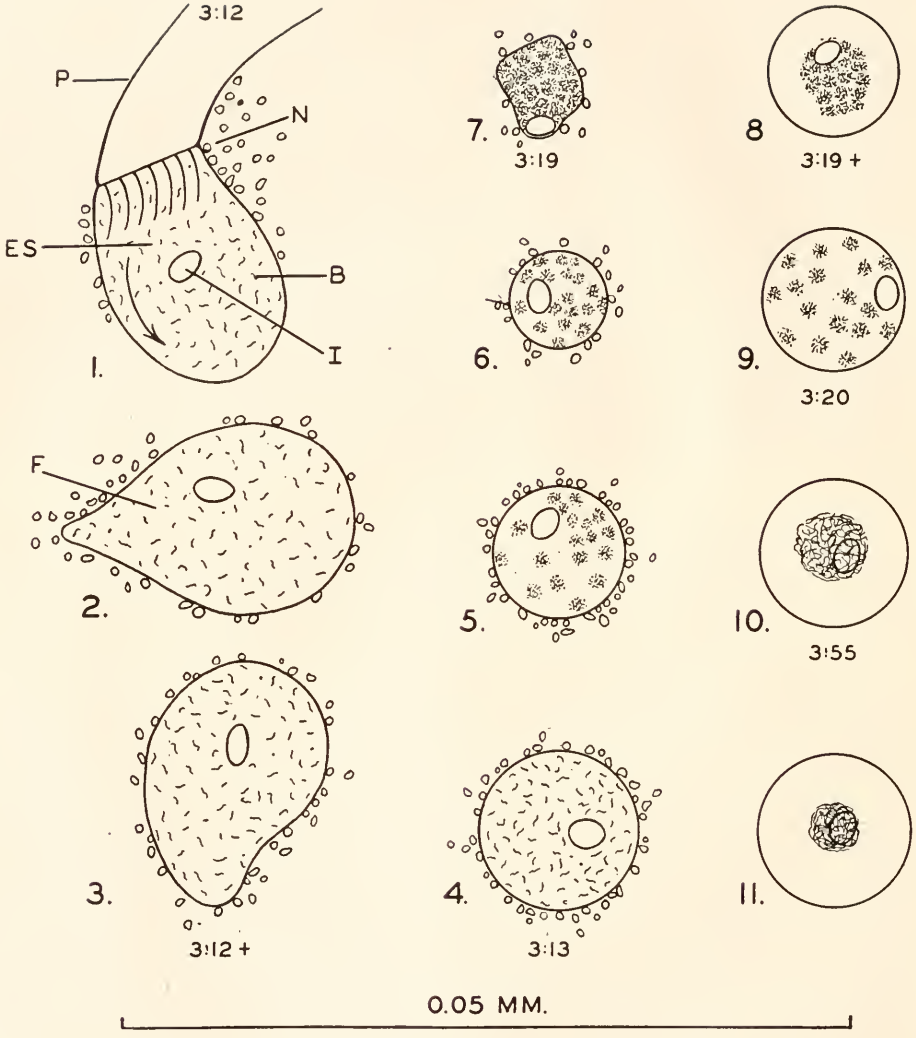

FIGURE 5. Camera outlines of a food-vacuole in a specimen of *Paramecium aurelia,* showing changes in form, size, and content, and changes in the position of neutral-red granules at the surface. *P,* pharynx; *ES,* esophageal sac; *F,* food-vacuole; *B,* bacteria; *I,* indigestible body; *N,* neutral-red granules; 3:12–3:55, time.

Note that the vacuole rapidly became spherical then decreased slowly but greatly in size, then increased rapidly and greatly. Note also that the numerous neutral-red granules at the surface of the vacuole all disappeared during its rapid enlargement and that the bacteria appear to have died and agglutinated during the decrease in size.

Changes in Shape and Size of the Food-Vacuole after it has left the Pharynx

Nirenstein (1905) contends that the food-vacuole in *Paramecium,* after it has left the pharynx, becomes spherical and decreases in size to about one-tenth and then increases until it is somewhat larger than it was originally. These contentions have been confirmed by others.

I made detailed observations concerning these phenomena in *Paramecium caudatum, P. nucleatum,* and *P. aurelia.* The results obtained show that they are fundamentally the same in these three species. The changes observed in a typical food-vacuole in *Paramecium caudatum* are illustrated in Figure 5.

This figure shows that the vacuole, after it had become free from the pharynx, rapidly became spherical and slowly decreased in size to nearly one-twentieth and then very rapidly increased until it was nearly as large as it had been originally. The figure indicates that as the vacuole decreased in size the bacteria in it died and aggregated in numerous small clumps, and that fluid passed out into the surrounding cytoplasm until there was practically none left, and the vacuole was so packed full of solid particles that the surface was very irregular. The figure indicates also that the numerous "neutral-red granules" at the surface of the vacuole when it left the pharynx, disappeared during its rapid enlargement. All these phenomena varied greatly in extent and time. For example, under some conditions there was no perceptible change in the size of the vacuoles at all. Details concerning some of them are given in the next section (see pages 55–57).

Mast and Bowen (1944) observed similar phenomena in the food-vacuoles in the Peritricha. They make the following statements in reference to the causes of the changes in size: "The loss of fluid, resulting in the decrease in the size, is probably due in part to difference in the osmotic concentration of fluids in the vacuoles and the cytoplasm [5] (that of the latter being higher than that of the former) and in part to inward pressure of the elastic membrane on the surface of the vacuoles, which was stretched by the pressure of fluid forced into them by the pharyngeal cilia. The inflow of fluid, resulting in increase in size, is probably entirely due to greater osmotic concentration within the vacuole than without. If this is true, the internal osmotic concentration must increase greatly during the time that the vacuole remains minimum in size. This could readily be brought about by transformation in the vacuole of osmotically inactive to osmotically active substance, for example, starch to sugar. In food-vacuoles which contain lactose, the gelatinous substance in them, referred to above, increases greatly in viscosity as the vacuoles decrease in size (as indicated by observations on Brownian movement) and then decreases greatly as they increase in size. The increase in viscosity is correlated with increase in acidity. It may well be that this increase in acidity causes chemical changes in the gelatinous substance which result in increase in osmotic concentration and that this

[5] This contention is strongly supported by the following observations: Mast and Bowen (1944) found that the food-vacuoles in *Vorticella similis* do not decrease in size if lactose in sufficient quantity is added to the culture fluid; and Fortner (1933) found the same in *Paramecium caudatum* in culture fluid to which *numerous* crushed bacteria had been added. In both of these observations the osmotic concentration of the fluid in the food-vacuoles was probably as high or higher than that of the surrounding cytoplasm, hence the retention of the water in them and no decrease in size.

in turn causes the rapid inflow of fluid from the cytoplasm which in turn, owing to decrease in acidity, causes the observed decrease in viscosity."

These statements are equally applicable to the food-vacuoles in *Paramecium.*

The Hydrogen-ion Concentration of the Content of the Food-Vacuole in Paramecium

It has been known for many years that after the food-vacuole in protozoa has been formed the hydrogen-ion concentration of its content increases considerably and then decreases again (Metchnikoff, 1889; LeDantec, 1890; Greenwood and Saunders, 1894; Nirenstein, 1905; et al.[6]). There is however marked diversity of opinion concerning the magnitude of these changes and the factors involved in producing them. Moreover, it has recently been maintained that in *Paramecium* the ingested substance becomes alkaline before the food-vacuole is formed, i.e., that there is in the vacuole a "preliminary alkaline phase."

The preliminary alkaline phase of the food-vacuole

Shipley and DeGaris (1925) made observations on specimens of *Paramecium caudatum* and *P. aurelia* in acid culture fluid to which phenol-sulfonphthalein (phenol red) had been added. They maintain that this fluid and the bacteria in it were distinctly yellow (acid), but that as it passed through the pharynx it became "faintly but distinctly pink . . . next to the wall of the pharynx", and that the forming food-vacuole was "filled with an alkaline [pink] fluid" which later became yellow (acid), and then pink (alkaline) again. They seem to hold that an alkaline substance is secreted by the wall of the pharynx.

Shapiro (1927) repeated the observations of Shipley and DeGaris and extended them to paramecia in neutral and in alkaline culture fluids. He says that in the neutral culture fluid "the vacuoles at first had a very light shade of pink" (alkaline), but that in the acid and the alkaline culture fluids there was no evidence of this. He concludes, however (p. 49), that "the observations made by Shipley and DeGaris of an alkaline stage at the beginning of the cycle has been verified." Dunihue (1931) asserts, on the contrary, that he was unable to confirm these observations.

I put into each of seven test-tubes a given quantity of fluid from a vigorous culture of *Paramecium caudatum* and added a different quantity of phenol red to the fluid in each, so as to produce a graded series of concentrations. All the solutions became distinctly yellow, and comparison with a series of buffers containing phenol red, showed that they were acid. After 24 hours the paramecia in the two strongest solutions were dead, but those in the rest of the solutions appeared to be in excellent condition. Some from each of these solutions were mounted and studied. They ingested fluid and bacteria in all, and the results obtained were the same in all.

[6] Engelmann (1879, p. 349) had previously observed ingested blue particles of litmus become red in *Paramecium aurelia, Stylonychia mytilus,* and *Amoeba diffluens.* But he did not know that this change in color was correlated with the content of the food-vacuoles. He assumed that the ingested particles of litmus were in direct contact with the cytoplasm and he therefore concluded that the observed change in their color demonstrated that the protoplasm is acid in these species.

Under a magnification of 400 (20 oculars and 20 objective) the stream of fluid in the pharynx was definitely yellow except a thin layer at the surface, i.e., a thin layer adjoining the wall of the pharynx, which was distinctly purplish pink. The fluid at the surface of the forming food-vacuole was also purplish pink, but that in the central portion was yellow, and the bacteria in it appeared to be the same in color as the fluid. After the vacuoles left the pharynx, they decreased slowly in size and the bacteria became intensely yellow, much deeper yellow than the fluid around them. Later the vacuoles increased rapidly in size, and the fluid in them became light purplish pink and the bacteria dark purplish pink.

These results are essentially in accord with the contentions of Shipley and DeGaris, and they appear to support their implication that the wall of the pharynx secretes an alkaline substance. However, on further examination of the paramecia in the culture fluids containing phenol red, I found that the surface of the content of the contractile vacuoles was also purplish pink, that is, that it was the same in color as the surface of the content of the pharynx, and I later observed the same purplish pink color in the pharynx and the contractile vacuoles in paramecia in culture fluid which did not contain any dye. Moreover, under a magnification of 1200 (20 oculars and 60 oil-immersion objective) there was no indication whatever of purplish or pinkish color in the pharynx or the contractile vacuoles, either in the paramecia in the culture fluid containing phenol red or in those in the culture fluid without any dye.

These results seem therefore to indicate that the purplish pink color observed in the lower magnification was due to refraction phenomena, not to change in acidity. It is consequently highly probable that the pinkish color observed in the pharynx and the forming food-vacuole by Shipley and DeGaris and Shapiro was not due to decrease in acidity.

I made numerous observations with several other indicator dyes, concerning a decrease in the acidity of the fluid in the pharynx in *Paramecium* but obtained no evidence of any. Moreover, Lund (1914) demonstrated fairly conclusively that in *Bursaria* there is a definite increase. That is, he found precisely the opposite from what Shipley and DeGaris maintain occurs in *Paramecium*. The evidence in support of a "preliminary alkaline phase" in the food-vacuoles in *Paramecium* is consequently negligible.

The Magnitude of the Changes in the Hydrogen-ion Concentration in the Food-Vacuole

Bozler (1924) was probably the first to attempt to measure the hydrogen-ion concentration of the content of the food-vacuole. He made observations on food-vacuoles in paramecia which had ingested yeast-cells stained with congo red; and he concluded that the hydrogen-ion concentration of their content increases approximately to pH 3. Nirenstein (1925) repeated and extended Bozler's observations and concluded that it increases nearly, if not quite, to pH 1. Neither of these investigators measured the subsequent decrease in hydrogen-ion concentration. Shapiro (1927) measured this and also the preceding changes. He made observations on paramecia in culture fluid containing respectively litmus, congo red, and phenol red, and concluded that in neutral culture fluid the acidity at first decreases to about pH 7.6 ("preliminary alkaline phase"), then increases to a maximum of

pH 4, and then decreases about to pH 7. Kalmus (1931) confirmed Nirenstein's conclusion.

I repeated and extended the preceding observations made with dyes and I also made observations on ingested crystals.

Ingested crystals

Neutral-red crystals were produced and tested for solubility and changes in color in relation to hydrogen-ion concentration as described in preceding papers (Mast, 1942; Mast and Bowen, 1944). Some fragments of these were added respectively to neutral and slightly acid or alkaline culture fluid containing paramecia on a slide and covered with a cover-glass supported on ridges of vaseline.

In some tests the culture fluid was taken from vigorous cultures which contained numerous bacteria; in others it was taken from old cultures which contained only a few bacteria, and in still others it was taken from a fresh culture which contained very few bacteria. Starch was added to the culture fluid in some of the tests. Observations were made on three different species of paramecia, namely, *caudatum*, *nucleatum*, and *aurelia*. The results obtained are essentially the same for the three species studied.

The paramecia fed freely in all the solutions used. Some of the food-vacuoles formed contained, in addition to the culture fluid ingested, only bacteria, others chiefly starch, and still others fragments of crystals, bacteria, and starch in various proportions. The fragments of crystals ingested ranged in size from irregular masses not much larger than the bacteria, to needle-like structures nearly half as long as the diameter of the paramecia.

The largest fragments invariably punctured the wall of the vacuole and passed out into the cytoplasm. Some of these were continuously observed under the oil-immersion objective for an hour or more. During this time they circulated several times around the body in the cytoplasmic stream and were then ejected. A few "neutral-red granules" became attached to the surface but no perceptible change occurred in the crystals, either in color or in form.

All the smaller fragments of crystals ingested remained in the food-vacuoles. These in most of the vacuoles did not change perceptibly and were ejected intact with the rest of the undigested substance. But those in some changed in color from brownish yellow to pink, and some of these rounded up and became nearly spherical. I did not see any disintegrate completely; but the fact that they rounded up shows that they became plastic and that they probably had partially dissolved.

These changes invariably occurred soon after the vacuoles had left the pharynx, but not until after they had decreased considerably in size. They occurred in vacuoles which contained only a few bacteria as well as in those which contained many, but not in all, and they did not occur in any vacuoles which were well filled with starch grains and contained but little fluid, and consequently decreased but little in size.

In acetate buffer solutions the crystals became pink immediately at pH 5 and lower, and dissolved in a few seconds. At pH 5.6 they became pink at the surface in 2 to 3 minutes and dissolved in 15 to 60 minutes. In hydrochloric acid, pH 4, they became pink at the surface immediately and dissolved in 10 to 20 minutes, but at pH 5 it required several minutes for them to become pink and more than 60

minutes for all to dissolve. Neutral-red crystals appear therefore to be considerably more readily soluble in acetate buffer solutions than in solutions of hydrochloric acid in distilled water. This difference seems to be correlated with the difference in the amount of buffers present.

The fact that the crystals became pink and, at least partially, dissolved in a few minutes in some food-vacuoles and did not change perceptibly in others shows that the maximum hydrogen-ion concentration of the content of the vacuoles varies considerably. This variation seems to be, at least in part, due to difference in the extent of the decrease in size of the vacuoles, for there was, as stated above, no change in the crystals in any of the vacuoles until after the vacuoles had decreased considerably in size.

The fluid in the food-vacuoles is doubtless well buffered and consequently resembles acetate buffer solution in this respect. Therefore, the facts that in acetate buffer solution the crystals become pink at pH 5.6 and that in many of the food-vacuoles they do not become pink indicate that the hydrogen-ion concentration of the fluid in these vacuoles does not reach pH 5.6. The results presented below show, however, that the maximum acidity reached in other vacuoles is very much higher.

Ingested dyes

The observations on ingested dyes were made as follows: A series of small test-tubes, containing some of the dye under consideration and appropriate buffer solution which differed by pH 0.2 in successive tubes, was prepared. Then culture fluid containing paramecia and bacteria was mounted between two parallel ridges of vaseline on a slide and dye or yeast-cells which had been stained by boiling in water containing dye, added. Then the preparation was covered with a cover-glass and the color of the content of the food-vacuoles formed, compared with that of the buffer in each of the test-tubes, and usually also with that of stained yeast-cells mounted respectively in buffer solution from each of the test-tubes. The hydrogen-ion concentration of the content of the food-vacuole was assumed to be that of the buffer and the stained yeast-cells in it, which it most nearly resembled.

Neutral red (pH 6.8, red—pH 8, auburn)

Three species of *Paramecium* were studied, *caudatum, nucleatum,* and *aurelia.* The results obtained were essentially the same in these three species.

Owing to the activity of the paramecia, it was usually impossible to keep a given food-vacuole under observation for more than a few minutes, especially under high magnification. I succeeded however in continuously observing one vacuole under 20 oculars and a 60 oil-immersion objective throughout practically its entire existence. Since these observations are exceptional and the results obtained concern various important phenomena aside from changes in hydrogen-ion concentration, they will be presented in detail.

The paramecium in which this vacuole formed, had been for 24 hours in culture fluid containing numerous bacteria and neutral red in low concentration. When it was discovered there were scattered through the cytoplasm about a dozen food-vacuoles and numerous small red granules or droplets often called "neutral-red granules." The vacuoles varied greatly in size and color. Some were pinkish red,

others yellow and still others various shades between. The red granules were much more abundant in the region around the distal end of the pharynx than elsewhere. They were especially abundant near the distal end of the aboral surface of the pharynx and the surface of the forming food-vacuole. There were so many granules on these surfaces that they appeared to form a continuous layer. Some of the formed food-vacuoles were also well covered with granules but others had none at all.

The food-vacuole under observation began to form about 3:11 P.M. It contained numerous bacteria and a small yellow neutral-red crystal which served admirably to distinguish the vacuole from others in the body. The bacteria were swimming actively and the entire content of the vacuole rotated rapidly, owing to the action of the cilia which projected from the pharynx into it. The vacuole left the pharynx at 3:12 P.M. and passed very rapidly nearly to the posterior end of the body. It turned approximately through 180° on the way, created violent currents in the cytoplasm and dragged numerous neutral-red granules after it. It was pear-shaped when it left the pharynx but became nearly spherical on the way to the posterior end of the body, i.e., in about one-fourth second. The bacteria and the fluid in it now appeared to be distinctly pinkish in color, but this was due to the red granules on the surface, for careful focusing showed that both the fluid and the bacteria in it were colorless. By 3:12½ P.M. the vacuole had decreased about one-fifth in diameter and the crystal had become spherical and crimson in color, but the bacteria were still colorless and active and the fluid was also still colorless. The crystal had apparently partially dissolved and had become plastic. At 3:15 P.M. the bacteria were motionless and faintly pink in color, but the fluid was still colorless. No further change had occurred in the crystal and the surface of the vacuole was still well covered with neutral-red granules. Some of these granules were seen to leave and others to approach the surface. *None passed into the vacuole.* By 3:17 P.M. the vacuole had decreased nearly one-half in diameter and had moved forward considerably. The bacteria had become deep pink in color, but no perceptible change had occurred in the fluid or the crystal or in the number of neutral-red granules on the surface. At 3:19 P.M. the diameter of the vacuole was only about one-fourth its original length. The bacteria were closely packed around the crimson crystal, forming a dark pink mass closely surrounded by the vacuolar membrane which was still well covered with red granules. A few moments later the vacuole began to increase rapidly in size. At 3:20 P.M. it had nearly reached its original size. During its enlargement the bacteria had become lighter in color, the fluid in the vacuole remained colorless and the neutral-red granules on the surface disappeared. By 3:23 P.M. the bacteria had become colorless, considerably larger and indefinite in outline and the crystal had become nearly colorless. No further changes in color occurred in the content of this vacuole. None of it became yellow. This was doubtless due to excessive dilution of the dye during the enlargement of the vacuole as indicated below. These results were confirmed by observations for shorter periods on many other food-vacuoles in this preparation.

In observations on food-vacuoles in paramecia in other preparations, some of which contained stained yeast-cells, the following was found: Under some conditions the content of the forming vacuole did not rotate, the bacteria were quiet and there was no Brownian movement. This indicates that the fluid in it was very viscous. The newly formed food-vacuoles were usually well covered with neutral-red gran-

ules, but occasionally there were only a few on the surface. Sometimes all the granules left the surface before the vacuole had decreased to minimum in size, but there was no indication that any entered the vacuoles. In preparations which contained but little neutral red, the neutral-red granules did not stain, but the bacteria in the food-vacuoles still became pink after they died. All but a few of the food-vacuoles observed were nearly spherical when they left the pharynx, and all these rapidly became spherical after they had left. The rest were spindle-shaped when they left the pharynx. Most of these rounded up very slowly after they had left and a few of them probably did not become spherical at all. Nearly all the spindle-shaped vacuoles observed were formed in paramecia which had been subjected for twelve hours or longer to relatively strong solutions of neutral red.

In most of the food-vacuoles observed a yellow hyaline layer formed at the surface, as the vacuoles enlarged after having decreased to minimum in size. This layer surrounded a red mass consisting of bacteria and other particles which gradually became uniformly distributed and distinctly yellowish in color. In the rest of the vacuoles observed, no layer formed at the surface, the distribution of the bacteria and other particles keeping step with the enlargement of the vacuoles; but the entire content of these vacuoles eventually became yellow, just as it did in those in which a layer had formed.

Ordinarily the food-vacuoles, after they had enlarged, remained intact until their undigested content was eliminated; but in a few instances two or more were seen to fuse, and some of the vacuoles thus formed were very large.

The results presents show that there is great variation in all the phenomena observed in the food-vacuole, but that the change in the acidity of its content is closely correlated with change in its size. They indicate that, as the vacuole decreases in size, the hydrogen-ion concentration of its content increases in some instances to more than pH 4 and then (as it increases in size) decreases approximately to pH 8. The colors of the neutral-red-containing buffers used in measuring the acidity of the neutral red stained content of the food-vacuoles, were however so indefinitely correlated with the acidity of the buffers that the results obtained are necessarily little more than crude approximations.

The Maximum Acidity of the Content of the Food-Vacuole

Congo red (pH 3, blue—pH 5, orange)

As stated above, congo red has been used by several investigators to measure the maximum acidity of the content of the food-vacuole in *Paramecium* and the conclusions reached differ greatly. In attempting to elucidate this diversity I extended the observations which have been made, using *Paramecium caudatum, P. nucleatum, P. aurelia,* and *P. trichium.*

In specimens of *P. caudatum* mounted in culture fluid containing numerous bacteria and congo red in excess, the following was found: The fluid in the newly formed food-vacuoles was light yellow, the undissolved particles of congo red, yellowish brown and the bacteria, colorless. As the vacuoles decreased in size after they had left the pharynx, the fluid in them decreased greatly in quantity and became light blue, the bacteria died and became dark blue and the particles of congo red became dark blue; then as the vacuoles increased in size, the fluid in them increased greatly

in quantity and became orange, after which the bacteria and the particles of congo red soon also became orange, and remained so until they were eliminated.

In some of these observations the paramecia, after having been a few minutes in the culture fluid containing bacteria and congo red, were transferred with as little fluid as possible, to a smear of polyvinyl alcohol on a slide and covered with a coverglass. These paramecia became so quiet that they could readily be studied under high magnification. Some of them contained food-vacuoles in which all the changes described above were very distinctly seen. When these food-vacuoles had become minimum in size the color of their content resembled that of buffer pH 3.2. These results therefore indicate that the maximum acidity of the content of the food-vacuoles in *Paramecium* is approximately pH 3.2 and that this is reached when the vacuoles have decreased to minimum in size. These observations were repeated, but yeast-cells which had been stained with congo red were added to the preparations.

The culture from which the paramecia were taken was pH 6.8. They were mounted in this fluid but NaOH was added to some of the preparations, so that a series was obtained which ranged from pH 6.8 to pH 9.8. The stained yeast-cells added were brilliant orange in color, and they remained so in all the preparations. The following results were obtained:

There was no perceptible change in the color in the ingested yeast-cells in any of the preparations until after the food-vacuoles containing them had left the pharynx and had decreased considerably in size. Then, in the preparations, pH 6.8–9, the yeast-cells in vacuoles which contained not more than about six, became sky-blue like buffer pH 3, as the vacuoles decreased in size and then, after the vacuoles had increased in size, brilliant orange again. If the food-vacuoles contained more than about six yeast-cells the extent of change in color from orange toward blue varied inversely with the number of cells in a vacuole. In those which were well filled with yeast-cells and consequently contained but little fluid, there was no appreciable change in color. These vacuoles decreased only slightly in size. The extent of change in color from orange toward blue, i.e., the increase in hydrogen-ion concentration, is therefore correlated with the number of yeast-cells in the vacuoles and the extent of the decrease in their size.

In fluid at pH 9.4 many of the paramecia did not feed, and those which did formed food-vacuoles which were considerably smaller and contained fewer yeast-cells than those formed in ordinary culture fluid. The cells in some of these vacuoles did not appreciably change in color; but those in others became definitely bluish, i.e., about like buffer pH 4. In fluid at pH 9.8 only a few of the paramecia fed and the food-vacuoles formed contained only a few yeast-cells. There was no perceptible change in color in any of these vacuoles, despite the fact that they contained only a few yeast-cells. This seems to show that the extent of change in color from orange toward blue depends upon the alkalinity of the fluid which is ingested with the yeast-cells. The fact, however, that no difference in the extent of change in color was observed in culture fluids which ranged from pH 6.8 to pH 9 indicates that this correlation is not very close.

In some of the observations paramecia were used which had been transferred successively through ten separate portions of fresh sterile culture fluid so as to eliminate nearly all the bacteria. The yeast-cells ingested in this solution changed in color as extensively as those ingested in solutions which contained numerous bacteria. The

increase in the acidity of the fluid in the food-vacuoles is therefore not closely, if at all, correlated with the number of bacteria in it.

There was no perceptible color in the fluid in the food-vacuole in any of the tests made with yeast-cells.

The results obtained with *P. nucleatum* and *P. aurelia* are essentially the same as those obtained with *P. caudatum,* but those obtained with *P. trichium* differed in that no change in color whatever was observed in the ingested yeast-cells. In this species the food-vacuoles usually contain only one yeast-cell when they leave the pharynx (never more than two) and very little fluid. They consequently change but little in size after they have left the pharynx. This is probably why there is no perceptible change in the color of the stained yeast-cells in them.

The results obtained with congo red therefore indicate that the maximum acidity of the content of the food-vacuole in *Paramecium* is approximately pH 3.2. This is in harmony with Bozler's conclusion. Nirenstein and Kalmus contend however, as previously stated, that the maximum acidity is much higher.

Nirenstein (1925) found, just as Bozler had, that ingested congo-red-stained yeast-cells become blue in some food-vacuoles in *Paramecium,* but he contends that Bozler's conclusion is erroneous. He put yeast-cells and coagulated egg albumen which had been stained orange with congo red, respectively into different concentrations of hydrochloric acid and found that, whereas a solution of congo red in N/1000 (pH 3) HCl is blue, neither the yeast-cells nor the egg albumen changed color in concentrations lower than N/30 (pH 1.47) and did not actually become blue until N/10 (pH 1) was reached. He consequently concluded that since stained egg-albumen and yeast-cells became blue in some of the food-vacuoles, the acidity of the fluid in those vacuoles must have been approximately pH 1. Kalmus (1931) repeated Nirenstein's observations and came to the same conclusion.

I added yeast-cells and congo red in excess to a series of Clark buffer solutions (pH 1–9) in test-tubes, heated them to boiling and then left them for 12 hours. At pH 2 and lower, the dye had precipitated, the fluid was very light purplish blue and the yeast-cells were not perceptibly stained. At pH 2.2 a few of the yeast-cells were slightly stained, light purple, the same shade as the fluid. At pH 3 the fluid was densely lavender and about one-half of the yeast-cells were strongly stained and the color was like that of the fluid. At pH 5 the fluid and all the yeast-cells were deep red. At pH 7 the fluid was the same color as at pH 5 but the yeast-cells were light orange. At pH 8 and higher the fluid was red but the yeast-cells were only slightly stained and light yellow in color. Congo red therefore appears to stain yeast-cells most readily in the neighborhood of pH 5.

Some of the red yeast-cells in buffer pH 5 were transferred to a series of buffers (pH 1–3) containing congo red and examined from time to time for two hours. At pH 1 and 1.2 the yeast-cells became densely sky-blue, at pH 1.4 purplish blue, at pH 2 and 3 definitely purple.[7]

Some of the red yeast-cells from the pH 5 buffer were added to culture fluid containing numerous paramecia. The yeast-cells were freely ingested. No change in color was observed in any of the food-vacuoles which were well filled with yeast-

[7] It is not clear why yeast-cells do not stain at all with congo red at pH 1 and 1.2 but become densely blue at these hydrogen-ion concentrations if they are first stained at higher concentrations.

cells, but in some of those which contained only a few, they unquestionably became as blue as those in buffers pH 1 and 1.2 and then, as the vacuoles increased in size, red again. In others they became purple of various shades so that all colors obtained in buffers pH 1–3 could be matched.

These results appear therefore to support Nirenstein's conclusion that the maximum acidity of the content of some of the food-vacuoles in *Paramecium* is approximately pH 1. The salt, protein and other errors for congo red are however so great that Clark (1928) and others maintain that the results obtained with it can be considered only as crude approximations. Moreover, in measurements made with a glass electrode, I found that congo red decreases the acidity of solutions and that in saturated solutions of congo red this decrease is great, i.e., from pH 3.06 to pH 6.61 in N/1000 HCl. Nirenstein's conclusion regarding the maximum acidity of the content of the food-vacuole, based on the assumption that congo red does not affect the acidity of solutions of hydrochloric acid, is consequently equivocal. I therefore repeated the experiments on congo red described above, with thymol blue and meta cresol purple, both of which have only very small errors and only slightly affect the acidity of solution to which they are added.

Thymol blue (*pH 1.2, red—pH 2.7, yellow*) and *meta cresol purple* (*pH 1.2, red—pH 2.8, yellow*)

In buffer solutions containing thymol blue, yeast-cells stain most readily at pH 3–5 and become lemon yellow. At pH 1–1.6 they do not stain perceptbily, but if yellow yeast-cells are put into these buffer solutions they become definitely pink of the same shade as the solutions.

Paramecia ingest the stained yeast-cells freely except in preparations in which the dye is too concentrated or the fluid too strongly acid. In every preparation studied the density of the yellow color of the yeast-cells in the forming food-vacuoles increased considerably. This is doubtless due to increase in the concentration of the dye, owing to impermeability of the vacuolar membrane to it and loss of water. After the food-vacuoles had left the pharynx, the yeast-cells in most of the vacuoles remained yellow but in some in nearly every preparation they unquestionably changed color and became as pink as those in buffer pH 1.4, and then as the vacuoles increased in size, yellow again.

The results obtained with meta cresol purple are essentially the same as those obtained with thymol blue. The ingested, stained yeast-cells remained yellow in most of the food-vacuoles, but in some they changed color as the vacuoles decreased in size and clearly became as pink as those in the buffer pH 1.4, and then yellow again as the vacuoles enlarged.

The results presented seem to demonstrate that while the maximum acidity of the content of the food-vacuoles in *Paramecium* differs enormously, it is at least as high as pH 1.4 in some of them. If this is true it is phenomenal, for culture fluid very much lower (even as low as pH 3.5) is instantly fatal to paramecia. The relatively high concentration of acid in the food-vacuole without injury to the adjoining cytoplasm is convincing evidence in support of the contention presented above that the vacuolar membrane is impermeable to acid.

The Maximum Alkalinity of the Content of the Food-Vacuole

Four dyes were used in the observations on the maximum alkalinity of the content of the food-vacuoles in *Paramecium,* nile blue (Grübler, 1915, pH 7, blue—pH 8, purple) ; phenol red (pH 6.8, yellow—pH 7.4, pink) ; brom thymol blue (pH 6, yellow—pH 7.6, blue), and cresol red (pH 7.2, yellow—pH 8.8, red). The observations made with congo red were repeated with each of these four dyes except that only two species (*P. caudatum* and *P. nucleatum*) were used and that the hydrogen-ion concentration of the culture fluid in which the paramecia were mounted, extended over a wider range. No difference was observed in the results obtained with the two species.

Nile blue

In the paramecia mounted in culture fluid containing nile blue, numerous food-vacuoles with many bacteria were formed in all the preparations except those which were so strongly alkaline that they were definitely toxic. These preparations were examined under low and high magnification from time to time for six hours. The content of the food-vacuoles was practically colorless until after the vacuoles had decreased considerably in size; then the bacteria in them became deep sky-blue in all the preparations and appeared to be dead, but the fluid remained colorless. Later the vacuoles increased in size, but there was no appreciable change in the color of either the fluid or the bacteria in them. There was no indication of a change to a purplish tint in the content of any of them. The buffer solutions containing Grübler's nile blue were sky-blue at pH 6.8, slightly purplish at pH 7.2, and distinctly purple at pH 7.6 and higher. The results presented, therefore, seem to show that the content of the food-vacuoles certainly did not become as alkaline as pH 7.6 in any of them, and probably not as alkaline as pH 7.2.

Yeast-cells stained with nile blue were mounted respectively in acid (pH 6.8) and alkaline (pH 8.6) culture fluids containing paramecia. The yeast-cells were freely ingested by the paramecia in all the preparations studied. In the acid culture fluid they were sky-blue and no perceptible change in color occurred as they passed through the body. In the alkaline culture fluid they were purple and they usually remained purple, but occasionally, if the food-vacuoles contained only one or two. they became blue as the vacuoles decreased in size and then purple again after they had enlarged.

Some of the blue yeast-cells were mounted in each of the buffer solutions in the series prepared. They remained blue in all below pH 7.6 and became only slightly purplish at pH 7.8. The results presented above indicate, therefore, that the content of some of the food-vacuoles formed in alkaline culture fluid became at least as alkaline as pH 7.8; but the fact that the yeast-cells became purple in only a very small percentage of the food-vacuoles shows that it rarely becomes as alkaline as this. However, the fact that the yeast-cells did not become purple in any of the vacuoles formed in acid culture fluid indicates that the maximum alkalinity of the content of the food-vacuoles is correlated with the hydrogen-ion concentration of the surrounding medium.

Phenol red

Paramecia can withstand a surprisingly strong solution of phenol red. If the solution is alkaline the cytoplasm becomes distinctly yellowish green. The bacteria in the culture fluid used containing phenol red were colorless and the fluid was yellow or pink, depending upon the hydrogen-ion concentration. In the food-vacuoles the bacteria, regardless of the hydrogen-ion concentration of the culture fluid, became dark yellow as the vacuoles decreased in size, but the fluid around them remained colorless. Then as the vacuoles enlarged the fluid and the bacteria became faintly but distinctly pink (pH 7.4) in some of them and colorless in the rest. These results are essentially in accord with those obtained by Shipley and DeGaris (1925).

Stained yeast-cells became distinctly yellow in the acid and distinctly pink in the alkaline culture fluids used. In the food-vacuoles the pink yeast-cells became yellow and the yellow ones remained yellow as the vacuoles decreased in size, the fluid remained colorless. In about five percent of the vacuoles, especially those which contained only a few yeast-cells, both kinds became faintly but distinctly pink in color similar to stained yeast-cells in buffer pH 7.4, as the vacuoles enlarged. In the rest they remained either yellow or became colorless. No definite correlation between the hydrogen-ion concentration of the surrounding medium and the changes in the color of the content of the vacuoles was observed.

These results indicate that the maximum alkalinity reached in the food-vacuoles varies greatly in different vacuoles but that it never exceeds pH 7.4. Phenol red is, however, not very satisfactory for the measurement of the hydrogen-ion concentration of the content of food-vacuoles; for the changes in color are rather indefinite and the color of buffer solutions is at best not closely correlated with their hydrogen-ion concentration.

Brom thymol blue

The bacteria in the culture fluid, both acid and alkaline, containing brom thymol blue were colorless. They were consequently colorless when they entered the food-vacuoles. No changes in color in them or in the fluid around them was observed as they passed through the body.

In the alkaline culture fluid used the stained yeast-cells became blue, and in the acid culture fluid yellow. The blue yeast-cells which were ingested became yellow almost immediately after the food-vacuoles left the pharynx, and in all the food-vacuoles which contained more than about four, they remained yellow until they were eliminated or became colorless; but in the food-vacuoles which contained fewer than about four they became (after the vacuoles had enlarged) greenish blue, about the shade assumed by stained yeast-cells in buffer pH 7 or 7.2. No difference in shade was observed in the acid and the alkaline culture fluids used.

These results indicate, therefore, that the maximum alkalinity attained by the content of the food-vacuoles is not greater than pH 7 or possibly 7.2, i.e., not so great as the results obtained with nile blue and phenol red indicate. The changes in the color of yeast-cells stained with brom thymol blue is, however, so elusive in the food-vacuoles that they indicate but little concerning the hydrogen-ion concentration of the fluid around them.

Cresol red

The bacteria in culture fluid containing cresol red were colorless when they were ingested, and no change in color was observed in them as they passed through the body.

The stained yeast-cells became light yellow in the acid culture fluid used and light pink in the alkaline. In the food-vacuoles they remained or became yellow as the vacuoles decreased in size and then, with a very few exceptions, either became colorless or remained yellow until they were eliminated, i.e., they did not change color after the vacuole had enlarged. In a few instances, however, the yellow yeast-cells assumed a light pink color, about like buffer pH 7.8.

It is evident that the results obtained with the four dyes used differ considerably. They seem to indicate however that the alkalinity of the content of some of the food-vacuoles is nearly, if not quite so high as pH 7.8 but that it is much lower in most of the vacuoles. They also indicate the maximum alkalinity varies somewhat with the acidity of the fluid ingested.

The Origin of the Acid and the Base in the Food-Vacuole

It is widely held that the acid and the base in the food-vacuole are secreted by the adjoining cytoplasm (Greenwood and Saunders, 1894; Nirenstein, 1905; Lund, 1914; Howland, 1928; Claff et al., 1941; and others). However, Mast (1942) concludes that in *Amoeba* the acid originates in the vacuoles and increases in concentration owing to impermeability of the vacuolar membrane to the acid and loss of water, and that the subsequent increase in alkalinity is due to diffusion of alkaline fluid from the cytoplasm into the vacuoles, that is, that "the cytoplasm secretes neither acid nor base." And Mast and Bowen (1944) conclude that in the Peritricha, "The increase in the acidity of the content of the vacuole is probably due to the production of acid, owing to metabolism in the peristome, the vestibulum and the pharynx and impermeability of the vacuolar membrane to organic acid, resulting in its retention and consequent concentration as the vacuole decreases in size." Mast and Bowen (p. 213) present strong evidence in support of their conclusion. This evidence applies equally well to the food-vacuoles in *Paramecium* and leads to the same conclusion. It should be emphasized, however, that secretion of acid by the cytoplasm adjoining *the vestibulum and the pharynx* is by no means ruled out.

The increase in alkalinity in the food-vacuole in *Paramecium* is doubtless brought about in the same way as it is in the Peritricha, i.e., by entrance of alkaline fluid from the cytoplasm. According to these views the cytoplasm adjoining the food-vacuoles does not secrete either acid or base.

The Function of the Changes in the Hydrogen-ion Concentration in the Food-Vacuole

It is maintained by Hemmeter (1896), Nirenstein (1925), Howland (1928), and Claff et al. (1941) that the organisms ingested by protozoa are killed by the acid in the food-vacuoles and that this is its primary if not its only function. There is, however, strong opposition to these contentions.

Fortner (1933) holds that in *Paramecium caudatum* the ingested organisms are killed by a toxic substance. He asserts that this substance is formed in the neutral-

red granules, and that these granules pass through the vacuolar membrane into the food-vacuole shortly after it has left the pharynx, and release the substance there.

These assertions are certainly not strictly valid, for as demonstrated above, the neutral-red granules do not enter the food-vacuole. It might be, however, that since some of them are in close contact with the vacuolar membrane when the food-vacuole leaves the pharynx, toxic substance diffuses from them into the vacuole and kills the ingested organisms. It is however far more likely that if toxic substance is involved in this phenomenon, it is secreted by the wall of the pharynx and taken up by the fluid which passes into the forming food-vacuoles, and that it there (owing to impermeability of the vacuolar membrane and loss of water) increases in concentration and consequently becomes lethal. At any rate, there is much evidence that the pharynx secretes substances which pass into the forming food-vacuole.

Fortner (1933) found that if numerous crushed bacteria are added to culture fluid containing live bacteria and paramecia the ingested bacteria are not killed, and that the vacuoles, as previously stated, do not decrease in size. This seems to show that death of the bacteria depends upon decrease in the size of the vacuoles. If this is true, it lends support to the hypothesis that the bacteria in the food-vacuole are killed by toxic substance which has been concentrated in the food-vacuoles owing to loss of water and impermeability of the vacuolar membrane to the toxic substance.

Mast (1942) in observations on *Amoeba* and Mast and Bowen (1944) in observations on the Peritricha present strong evidence that the acid in the food-vacuoles in these protozoa does not become sufficiently concentrated to kill the ingested organisms and the results presented above seem to show that this also obtains for most of the food-vacuoles in *Paramecium*. Mast concludes that in *Amoeba* the primary lethal factor in the food-vacuole is reduction of oxygen, owing to respiration and loss of water, and Mast and Bowen accept this view in reference to the Peritricha, but they present no evidence in support of it and none was obtained in the present observations on *Paramecium*.

No one has observed any indication of digestion in the food-vacuole during its acid phase. Nirenstein (1905) thinks, however, that there are invisible preliminary changes. However this may be, it is practically certain that the osmotic concentration of the fluid in the vacuole increases greatly during this phase, and it may well be that this is caused by hydrolysis of complex compounds, owing to the presence of acid, and that this causes the rapid inflow of fluid from the cytoplasm, which probably contains digestive enzymes. If this is true, the acid in the food-vacuoles obviously functions, at least indirectly, in digestion. There are consequently several hypotheses concerning the function of the acid in the food-vacuole but there is not much evidence in support of any of them.

It has been conclusively demonstrated that digestion in the protozoa takes place largely, if not entirely, in the food-vacuoles during the alkaline phase, but since this phase seems to be merely the result of inflow of fluid from the cytoplasm, digestion is not correlated with anything in the nature of secretion of alkaline substance.

Digestion

Nirenstein (1905) made detailed observations on bacteria, yolk granules, fat globules, and starch grains in the food-vacuoles in *Paramecium caudatum*. He says he observed no indication of digestion in any of these substances until after the fluid

in the vacuoles had become alkaline, but that after this had occurred the bacteria and the yolk granules gradually decreased in number and finally disappeared entirely and some of the starch grains corroded. He was at this time in doubt about the fate of the fat globules, but he later (1910) presented evidence which strongly indicates that fat is hydrolyzed in the food-vacuoles.

Nirenstein's views concerning the time of digestion in the food-vacuoles in *Paramecium* and the digestion of proteins have been adequately confirmed; his contention that paramecia digest starch is supported by results obtained by Pringsheim (1928) and Zingher (1933) in observation on *Paramecium caudatum;* and his contention that they digest fat is indirectly supported by results obtained by Dawson and Belkin (1929), Mast (1938), and Wilber (1942) in observations on *Amoeba* and *Pelomyxa.*

I repeated Nirenstein's observations and obtained results which are in harmony with his. It should be emphasized, however, that in my experiments only a very small proportion of the starch (wheat and potato) ingested by the paramecia was digested. Observations by Greenwood (1886), Meissner (1888), and Mast and Hahnert (1935) indicate that this also obtains for *Amoeba.* Indeed, it is doubtful whether in this organism any starch is digested except that which is in other organisms which have been ingested.

The Origin of the Digestive Enzymes and the Function of the Neutral-Red Granules and the Mitochondria

The fact that protein, starch and fat are digested in the food-vacuoles in *Paramecium* shows that these vacuoles contain peptidase, amylase, and lipase, and the fact that digestion does not begin until after the fluid in the vacuole has become alkaline shows that these enzymes are active in a basic medium. The questions now arise as to where these enzymes originate and how they get into the food-vacuoles.

Prowazek (1898) who, in observations on paramecia, discovered the neutral-red granules and their tendency to aggregate at the surface of the forming food-vacuole, maintains that they contain digestive enzymes. He consequently called them "Fermentträger". He offers no explanation as to how the postulated enzymes in the granules get into the vacuole, but Nirenstein (1905) who accepts Prowazek's contentions, holds that the granules pass through the vacuolar membrane and carry the enzymes into the vacuole. He asserts that, in paramecia in culture fluid containing neutral red, he observed red granules appear in food-vacuoles shortly after they had left the pharynx. He consequently concluded that the red granules in the food-vacuoles came from the cytoplasm and passed through the vacuolar membrane into the food-vacuoles and that the enzymes in the vacuoles are carried in by these granules.

These conclusions are supported by Rees (1922), Bozler (1924), Fortner (1926), Volkonsky (1929), Müller (1932), and MacLennan (1941, p. 129). All these investigators hold that in *Paramecium* the neutral-red granules actually pass through the vacuolar membrane and carry enzymes into the food-vacuoles. None of them, however, maintains that he saw this. Their contention is based largely on Nirenstein's observation of the appearance of red granules in the food-vacuoles. It is, however, obvious that these granules may have entered the food-vacuoles by way of the pharynx. The evidence in support of the contention that neutral-red

granules pass from the cytoplasm into the food-vacuoles in *Paramecium* is therefore very weak. There is, however, evidence which strongly indicates that in some other organisms neutral-red staining substance actually passes from the cytoplasm into the food-vacuoles.

MacLennan (1936) asserts that in *Ichthyophthirius* neutral-red staining substance becomes attached to particles of food which are naked in the cytoplasm, that these particles aggregate, and that a membrane then forms, enclosing them, together with some fluid, in a vacuole. He thinks that the neutral-red staining substance contains digestive enzymes. Hopkins and Warner (1946) hold similar views regarding the food-vacuoles in *Entamoeba histolytica* and they maintain, moreover, that they actually saw globules pass through the vacuolar membrane into and out of the food-vacuoles.

The idea that granules or globules carry enzymes into the food-vacuoles, is consequently widely held. There is, however, evidence which very strongly indicates that this does not obtain in *Paramecium.*

Khainsky (1911) studied the food-vacuole of *Paramecium caudatum* in fixed and stained specimens and in living specimens. He obtained no evidence which indicates that granules or globules pass from the cytoplasm into the food-vacuoles. He maintains, however, that neutral-red staining globules form in the vacuole and pass out through the vacuolar membrane. Koehring (1930) made extensive observations on neutral-red granules in various protozoa. She concludes that they function as enzyme carriers in all, but that they do not enter the food-vacuoles. Referring to *Paramecium caudatum* she says (p. 67): "As the new vacuole is being formed they [the neutral-red granules] gather at the membrane, bombarding it like hailstones, but making no impression on the firm surface. Then as this vacuole flows away attached by its canal . . . some of the granules leave this vacuole and return to the next, which is already in the process of formation. Those left continue bombardment of the vacuole as the pink color slowly forms within."

Dunihue (1931) asserts that in *Paramecium caudatum* the surface of the forming food-vacuoles becomes "closely packed" with neutral-red staining globules "which remain firmly attached throughout most of the digestive period" and do not enter the vacuoles.

As stated in a preceding section, I made under most favorable conditions intensive observations on the neutral-red granules at the surface of food-vacuoles throughout their entire existence and obtained no evidence indicating that visible granules or globules of any kind ever pass through the vacuolar membrane into or out of the food-vacuoles in *Paramecium.* What, then, was the origin of the red granules observed by Nirenstein in the food-vacuoles in *Paramecium?*

These granules doubtless originated in substance ingested by the paramecia. At any rate it has been clearly demonstrated that this occurs in *Amoeba proteus* (Mast and Hahnert, 1935; Mast and Doyle, 1935). It can therefore be concluded that the neutral-red granules found in the cytoplasm in *Paramecium* do not enter the food-vacuoles. This does not, however, demonstrate that they are not involved in digestion. If they contain enzymes it may be that in aggregating at the surface of the food-vacuoles the concentration of enzymes is increased and that this facilitates their diffusion into the vacuoles. The facts, however, that there is a marked flow of fluid out of the vacuole during the entire time that the aggregation of these granules is prominent and that most of the granules usually leave the surface of the vacuoles

long before digestion begins, seem to militate against this possibility. Moreover, Hall and Dunihue (1931) and Mast and Bowen (1944) found that in *Vorticella* and other Peritricha the neutral-red granules do not aggregate at the surface of the food-vacuoles, and Mast (1926) found that in *Amoeba* there are none. They are therefore certainly not functional in digestion in the latter and very probably not in the former. The evidence in support of the contention that they function in digestion in *Paramecium* is therefore negligible. What, then, is their function in this organism?

Fortner (1926, 1928) maintains that they originate in the cytoplasm adjoining the nucleus, that they contain protein and fat, and that they decrease greatly in number during starvation. If these contentions are valid, the composition of the granules and their disappearance seem to suggest that they are reserve food; but, if this is what they are, why do they originate near the nucleus, and why do they aggregate at the surface of the food-vacuole during its formation? The function of the neutral-red granules in *Paramecium* is apparently still decidedly hazy.

Horning (1926a) contends that in *Amoeba sp.* janus-green staining bodies (mitochondria) aggregate among particles of food in the cytoplasm and that a membrane then forms and encloses the food and the mitochondria in a vacuole. He says that in *Paramecium sp.* mitochondria "are extruded from the cell protoplasm [through the vacuolar membrane] into the food vacuoles" during the alkaline phase. He concludes that the mitochondria in these protozoa function in carrying digestive enzymes to the ingested food. Volkonsky (1934) does not agree with Horning. He maintains that the mitochondria do not enter the food-vacuoles and that they do not take part in digestion in protozoa.

As stated above, I was unable to see any particles pass from the cytoplasm into the food-vacuoles in *Paramecium.* Hall and Nigrelli (1930) could not obtain any evidence of mitochondria in the food-vacuoles in *Vorticella,* and Mast and Doyle (1935) in an intensive study of the movement of the mitochondria in *Amoeba proteus* under very favorable conditions concluded that they do not pass into the food-vacuoles. They found that the mitochondria move about in the cytoplasm freely and often come in contact with the surface of the food-vacuoles in all stages of development and at times form aggregations at the surface, but that none pass into the vacuoles. These observations and others led them to conclude that the mitochondria in *Amoeba* "probably function in transferring substances from place to place in the cytoplasm."

Holter and Kopac (1937) maintain that in centrifuged specimens of *Amoeba proteus,* the mitochondria are in a fairly well defined stratum but that the enzyme, peptidase, is not stratified. They consequently conclude that this enzyme is not associated with mitochondria. However, Holter and Doyle (1938) in similar tests found that amylase and mitochondria in *Amoeba* tend to accumulate in the same stratum. These results seem to indicate that this enzyme is attached to the mitochondria. The authors do not, however, hold that this association is proved.

The evidence in support of the contention that the mitochondria and the neutral-red granules in *Paramecium* are enzyme carriers is therefore, as yet, far from convincing. Where, then, do the digestive enzymes in the food-vacuoles come from?

Some doubtless come from the organisms and other substances which have been ingested and the rest are, I think, as previously stated, carried from the cytoplasm into the vacuoles with the fluid that enters during the rapid enlargement of the vacuoles at the beginning of the alkaline phase. Where and how the enzymes in the

cytoplasm originate is not known. Fortner (1926), as stated above, maintains that the neutral-red granules are formed in the cytoplasm adjoining the nucleus. He holds that the enzymes are closely associated with these granules and consequently concludes that they also originate in this region. The validity of the contention that the enzymes are closely associated with the neutral-red granules is, however, very doubtful. The evidence in support of Fortner's conclusion is, therefore, very weak. It is known however that in *Chilomonas paramecium* enzymes are synthesized in the protoplasm, for this organism can be grown on known simple chemical compounds (Mast and Pace, 1933), but how and where they are synthesized and what they are, chemically, is not known, and this can also be said of the enzymes in other protozoa.

Summary

1. The feeding apparatus in *Paramecium* consists of a shallow ciliated groove, a ciliated tube which leads into the body, and a bundle of fibers (esophageal fibers) which extend from the tube nearly to the posterior end of the body. The tube is composed of an outer part (the vestibulum) and an inner part (the pharynx).

2. Paramecia ingest all sorts of small particles, but more digestible than indigestible ones. Selection takes place in the vestibulum and the proximal end of the pharynx.

3. In forming a food-vacuole, the cilia in the pharynx force fluid with particles in suspension against the membrane over the distal opening of the pharynx, producing a sac, the esophageal sac.

4. As the esophageal sac enlarges, the particles in suspension in it become greatly concentrated, owing largely, if not entirely, to the passage of water out through the membrane into the cytoplasm.

5. A portion of this sac is constricted off, as a food-vacuole, probably by the action of the esophageal fibers.

6. The initiation of the constriction of the sac is probably due to periodicity in the constrictive action of the fibers, the size of the sac, and the composition of its contents.

7. There is much variability in the size of the food-vacuoles. This is correlated with the quantity and the quality of the particles in the surrounding fluid, the chemical composition of this fluid, the rate of ingestion, the rate of loss of water from the esophageal sac, and the length of the intervals between consecutive constrictions of the esophageal fibers. The frequency of formation of food-vacuoles is correlated with the quantity and the quality of the particles in the surrounding fluid and the acidity, and the temperature of this fluid. The shape of the food-vacuoles depends largely, if not entirely, upon the viscosity of their content.

8. After the food-vacuole has left the pharynx it passes rapidly on a fixed course toward the posterior end of the body, and slowly on a varied course to the anus. The former is probably due to the action of the esophageal fibers; the latter is due to cyclosis.

9. On its course through the body, the food-vacuole usually decreases greatly in size, and the acidity of its content increases greatly; then it enlarges very rapidly and the acidity of its content decreases greatly. The extent of these changes varies enormously. Under some conditions there are no perceptible changes; under others

the acidity in some vacuoles increases to a maximum at least as high as pH 1.4 and then decreases approximately to pH 7.8.

10. There is no "preliminary alkaline phase" of the food-vacuole.

11. The change in acidity is definitely correlated with change in size. The changes in size are due to difference between internal and external osmotic concentration and the action of the stretched vacuolar membrane. The increase in acidity is probably due to secretion of acid by the cytoplasm adjoining the vestibulum and the pharynx and to impermeability of the vacuolar membrane to hydrogen-ions, and loss of water. The decrease in acidity is due to entrance of alkaline fluid from the cytoplasm.

12. The increase in acidity probably causes hydrolysis and thereby increase in osmotic concentration resulting in inflow of fluid containing digestive enzymes.

13. Death of ingested living organisms is probably largely due to toxic substance produced by the pharynx and concentrated in the food-vacuole, owing to impermeability of the vacuolar membrane to it, and loss of water.

14. Paramecia digest protein, fat, and starch. Digestion takes place during the alkaline phase of the food-vacuole. The enzymes involved originate in the cytoplasm and are carried into the food-vacuole by the cytoplasmic fluid which enters during its rapid enlargement.

15. The neutral-red granules and the mitochondria are probably not involved in digestion.

16. All these phenomena are essentially the same in the four species studied, namely *P. caudatum, P. nucleatum, P. aurelia,* and *P. trichium.*

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POISONING AND RECOVERY IN BARNACLES AND MUSSELS [1]

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Introduction

Previous investigations have shown that the prevention of the attachment of organisms to ships' hulls by antifouling paint is related to the rate of solution of the toxic material, and hence, to its concentration in the sea water at the paint's surface (Ketchum, Ferry, Redfield, Burns, 1945). The present investigation was undertaken to test the effectiveness of different metallic salts as poisons for two important types of fouling organisms and to evaluate them under conditions in which the concentration and ionic form of the toxic materials were accurately known. In earlier attempts to test lethal action, supposedly toxic materials have been incorporated in paints applied to test-panels or to the inside surfaces of containers and the degree of fouling resistance noted, but in most cases no adequate measure was made of the concentrations of the toxics which were actually emitted by the paint. In other tests, fouling organisms were placed in containers of sea water to which known amounts of toxics had been added but without adequate knowledge of the ionic transformation of the toxics which ensue in the presence of sea water (Bray, 1919; Edmondson and Ingram, 1939; Orton, 1929–30; and Parker, 1924).

We proposed, therefore, to conduct direct tests of the action of various individual metals in graded concentrations and in different chemical forms, on two important types of fouling organisms; namely, barnacles and mussels. We desired also to investigate the relationship between the concentration of various toxics and the exposure time necessary to kill, in order to ascertain the most efficient concentration of the poison for the destruction of fouling organisms.

A second purpose was to follow the course of poisoning and recovery in barnacles and mussels when copper was used as the poison. It was desired to measure the amount of copper in the tissues and to discover whether death from copper poisoning was brought about specifically by the accumulation of a certain amount of the poison in the tissues or by the entrance of the copper into the body at a certain rate or for a critical period of time. Finally, it was desired to ascertain from how great a dose of copper an animal could recover, and to learn whether, after recovery, the animal was more or less susceptible to subsequent exposures of the toxic.

I am indebted to Mrs. Dayton Carritt for assistance in carrying out the experiments, to Mrs. Barbara Mott Ferry for chemical analyses, to Dr. John Ferry and Dr. Gordon Riley for furnishing toxics, and to Mr. Porter Smith for technical assistance. I am indebted to the University of Miami for the use of facilities at the

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[2] Contribution Number 360.

Marine Laboratory. I also wish to thank Dr. Walton Smith and Dr. Charles Pomerat for valuable cooperation.

Observations on Adult Barnacles and Mussels

The effectiveness of various poisons was investigated first using the barnacle as a test animal, since the barnacle is generally the most serious offender as a fouling organism. Adult barnacles were employed for the majority of the experiments at Woods Hole, but one set of tests was conducted on nauplii which had been discharged in a laboratory tank. Although attachment of the barnacle takes place previous to the adult stage, there is evidence that a poisoned barnacle is less securely attached than a normal animal and may be more easily dislodged. Special interest centers about the poisoning of the cyprid, since this is the attaching stage. In view of the fact that cyprids could not be obtained in sufficient numbers at Woods Hole, experiments on this stage were conducted at Miami Beach, Florida. Mussels, which are of relatively large size, were found advantageous for other tests, especially those involving the copper assay of living tissue.

Source and preparation of material

Certain of the present tests were carried out with *Balanus balanoides* growing on stones obtained near low tide mark at Woods Hole. In later tests *Balanus eburneus* was employed, obtained through the kindness of Dr. Stanley Cobb, from a wharf in Cotuit Harbor. Parts of the wharf were removed and transported to the laboratory tanks. The barnacles were kindly identified by Dr. Richard McLean.

For the experiments with *B. balanoides* the material was collected on the same day that the tests were begun. Single stones bearing 10 to 30 barnacles (ranging from 3 mm. to 10 mm. in diameter) were placed in individual beakers or culture dishes containing from 250 cc. to 500 cc. of sea water with or without toxic. For the work using *B. eburneus,* a stock of animals attached to fragments of wood was maintained in large battery jars provided with running sea water. In preparation for the tests it was necessary to split and saw the pieces of wood into blocks of convenient dimensions. Blocks about 4 cm. to 6 cm. square, each bearing 10 to 20 barnacles ranging from 4 mm. to 20 mm. in diameter, were similarly placed in individual containers. In other experiments larger blocks bearing several hundred barnacles were placed in battery jars of 8 liters capacity or more.

With both species of barnacles it was found that when the sea water was renewed daily, the animals in the individual containers remained in a healthy condition for several weeks. There were evidently enough food particles in the sea water supplied to provide a maintenance diet. Faecal material was produced in abundance but the barnacles grew little, if at all.

The mussels employed were the common species in the Woods Hole region, *Mytilus edulis,* obtained from the dock and float of the Oceanographic Institution. The mussels were placed individually or in batches in culture dishes or large battery jars. The animals began attaching their byssus threads to the bottoms or sides of the containers almost immediately. If the sea water was renewed each day, the mussels continued in healthy condition indefinitely, apparently obtaining sufficient nutriment from the food particles present in the sea water.

Behavior during poisoning

When barnacles were placed in the toxic media, they opened their shells and began raking or filtering the water with their cirri as soon as did the controls in fresh sea water. Ordinarily this took place within a few minutes. If the concentration of the poison was such as to kill the barnacle within two or three days, the animals would begin to show signs of bad condition after about 12 hours. The first symptom was the slowing down of the beat of the cirri and soon thereafter their sweep became reduced in extent. As poisoning progressed, the movement of the cirri became still slower and more curtailed until they opened to only one-half or one-quarter of their usual scope. When the poisoned barnacle stopped filtering, it tended to come to rest with the cirri half extended, in contrast to the healthy animal in which the cirri are drawn completely in and the shell tightly closed during rest. In the poisoned animal, the shell could at first be caused to close by prodding it with a needle. After further poisoning, when no response could be elicited by stimulation with the needle, the barnacle was considered dead. The following symbols are used in the tables to designate these conditions:

++++	normal rapid sweep of cirri
+++	full sweep but slow
++	incomplete sweep
+	open, inactive, but reacts to touch
0	dead

If the barnacle was replaced in fresh sea water (preferably running sea water) within a day of the time it reached the next to last condition (+), it would usually recover completely its full activity after a few days.

In the case of the mussel, the change in condition as poisoning progressed was less easily observed. In the higher concentrations of the toxics, the animals failed to attach themselves to the walls of the containers and tended to remain closed for long periods. When placed in weaker solutions, however, many of the mussels became strongly attached and remained with their shells open to the normal extent to allow the inflow and outflow of water. In either case, after poisoning had progressed to a certain point, the valves were found gaping wide open. When this condition was first reached, the animal responded to prodding by closing slowly. A day later, or less, no response would follow such stimulation and the animal was considered dead. Once the mussel reached the stage in which it gaped open more than normal, though it might still react, it would not recover when placed in fresh sea water. In fact, mussels were frequently observed to die many days after having been transferred from the toxic solution into fresh sea water although outwardly they appeared to be in normal condition in the interim.

Toxicity of various metallic salts to barnacles

The toxicity of the following metals was investigated: copper, mercury, silver, and zinc. The last three of these was tested as the ions which resulted from the introduction into the sea water of $HgCl_2$, $AgSO_4$, and $Zn(NO_3)_2$ respectively. The copper, on the other hand, was tested not only in the form of basic cupric carbonate, but also as cupric citrate, cupric tartrate, cupric salicylate, and cupric para amino-

benzoate. The interest in these other compounds of copper lies partly in the possibility of revealing important differences in toxicity. Of further significance is the fact that these substances are much more soluble in sea water than is basic cupric carbonate. Therefore, when copper is supplied in the form of these more soluble ions, it is possible to establish a high concentration of the toxic in the water adjacent to the surface from which the material is free to dissolve.

Individuals of *Balanus balanoides* were placed in beakers or jars containing graded concentrations of toxics in sea water with a control for each series. Since the media were renewed each day, the danger of significant changes in concentration due to such causes as adsorption was minimized. The minimum concentrations necessary to kill 90 per cent of the barnacles after continuous exposures of two days and five days are indicated in Table I. Among the various forms of copper, the

TABLE I

The concentrations of various metallic salts necessary to kill 90 per cent of adult barnacles after continuous exposures for the indicated periods. Concentrations are expressed as milligrams of metal per liter of sea water

	Toxic	Concentration lethal in 2 days	Concentration lethal in 5 days
		mg./liter	mg./liter
Series I			
	Basic Cu Carbonate	0.35	—
	Basic Cu Carbonate	0.48	—
Balanus balanoides	Cu Citrate	0.60	0.18
from	Cu Citrate	0.60	0.30
Woods Hole harbor	Cu Tartrate	0.58	0.17
	$HgCl_2$	1.0	0.5
	Ag_2SO_4	0.4	0.2
	$Zn(NO_3)_2$	32.	8.0
Series II			
	Basic Cu Carbonate	0.28	0.14
Balanus eburneus	Cu citrate	0.55	0.14
from	Cu salicylate	0.90	0.45
Cotuit harbor	Cu para aminobenzoate	—	0.50

basic cupric carbonate was the most effective but the concentrations of the citrate and tartrate necessary to kill were only slightly greater. The toxicity of silver as silver sulphate was of the same order of magnitude but somewhat greater concentrations of mercury as mercuric chloride were required. The effectiveness of zinc, on the other hand, was very much less than any of the other substances tested (*cf.*, Riley, 1943).

Experiments with *Balanus eburneus* indicated similarly that the lethal action of cupric citrate is somewhat less than that of basic cupric carbonate although the magnitude of the difference is not great. The toxicity of cupric salicylate and cupric aminobenzoate were somewhat less than the citrate.

In view of the fact that the toxicity of cupric citrate was found to be almost as great as that of basic cupric carbonate, and since stock solutions of higher and less

variable concentrations could be maintained, the tests which follow were conducted with the citrate.

Relation between concentration of cupric citrate and killing time

Barnacles

With the higher concentrations of cupric citrate in which killing occurred within a few days, adult barnacles all succumbed at about the same time, but at the lower concentration it was difficult to determine accurately when the barnacles should be considered dead. At still greater dilutions of the toxic, it was found that most of the animals lived on in a feeble condition for two or three weeks or more. Furthermore, there was considerable variation from experiment to experiment, which was no doubt due not only to individual differences in the batches of barnacles, but also

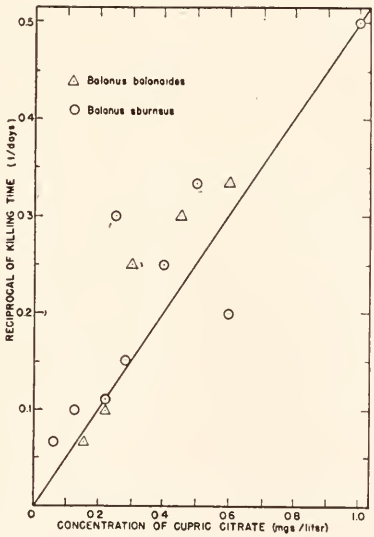

FIGURE 1. The relation between killing time and concentration of toxic for two species of barnacles.

to unavoidable changes in the seasonal condition and in the age of the animals. No consistent trend appeared in relation to the latter conditions except that barnacles in the nauplius stage were killed much more quickly than the adults, as noted below.

For both *B. balanoides* and *B. eburneus,* the killing time ranged between two and five days for concentrations of 0.60 to 1.00 mg. Cu/liter. At 0.22 mg./liter the animals succumbed after ten days. At concentrations of 0.06 mg./liter and less, the animals remained alive for two weeks or more. When the reciprocal of the killing time was plotted against the concentration, as in Figure 1, a roughly linear relation was found. The approximate linearity of this relation indicates that the rate of action of the poison is directly proportional to its concentration.

The results of a series of tests on the poisoning of nauplii, made on the day of their release from the brood pouch, are shown in Table II. They indicate that the

TABLE II

Effect of concentration of cupric citrate on the survival of nauplii of Balanus eburneus. August 25–27, 1942

Concentration mg. Cu/liter	After 22 hrs.	After 29 hrs.	After 48 hrs.
1.00	all dead	—	—
0.50	all dead	—	—
0.25	all dead	—	—
0.13	75% alive	all dead	—
0.06	75% alive	50% alive	1% alive
0.03	100% alive	95% alive	5% alive
Control	100% alive	95% alive	5% alive
Control	100% alive	95% alive	10% alive

nauplii are killed after much shorter exposure than is the case with the adults. The test was not very satisfactory because the controls lived for only about two days.

Mussels

The experiments on the mussel, *Mytilus edulis,* indicate that it is much more sensitive to cupric citrate than the barnacle (Tables III and IV). A half-day exposure to 0.55 mg. Cu/liter was sufficient to kill the three mussels in this concentration. One animal in each of the experiments succumbed following a half-day exposure to a concentration of only 0.14 mg. Cu/liter. With adult barnacles, exposures of two days and five days are required for killing at these concentrations. In these tests, and in many others with mussels, death did not ensue until several days after the animals had been transferred to fresh sea water. In one case the mussel did not die until the tenth day following a half-day exposure to the poison. In most cases the animals showed no outward sign of unhealthy condition in the interim.

TABLE III

Relation of killing time to concentration of cupric citrate in the mussel, Mytilus edulis. In each test three animals (about 3 cm. long) were placed in each toxic solution for the periods indicated and then transferred to fresh sea water. Started August 6, 1942

Concentration mg. Cu/liter	Time in toxic days	Killing time days
0.55	½	4, 8, 10
	1	2, 3, 4
	2	2, 3, 3
	3	2, 3, 3
0.14	½	4, *, *
	1	3, 3, 4
	2	3, 4, 4
	3	3, 4, 4

* Still alive when experiment terminated on August 18.

TABLE IV

Relation of killing time to concentration of cupric citrate in the mussel, Mytilus edulis. In each test two animals (about 3 cm. long) were placed in each toxic solution for the periods indicated and then transferred to fresh sea water. Started August 10, 1942

Concentration mg. Cu/liter	Time in toxic days	Killing time days
0.14	½	5, *
	1	3, 4
0.08	½	*, *
	1	*, *
	2	6, 8
0.04	½	*, *
	1	*, *
	2	*, *
	3	8, *
0.02	1	*, *
	2	*, *
	4	*, *

* Still alive when experiment terminated on August 20.

Accumulation of copper in barnacle tissues during poisoning and recovery

Several series of experiments were undertaken to determine the extent to which copper accumulated in the tissues of the barnacles subjected to solutions of cupric citrate. Since the shell material of the barnacle is several times greater in volume and many times greater in dry weight than the soft tissues, a technique was developed for the copper assay of both constituent parts of the animal separately. The number of barnacles used for a single analysis varied from 15 to 40 with the size and expected copper content. In the earlier experiments, the animals were chiseled free from the substratum, but in so doing all the fluid material was lost, and bits of broken shell were sometimes included with the soft parts. In the later experiments the soft tissues were taken from the shell without disengaging the animals from the substratum by removing the operculum and picking out the soft parts with forceps. The fluid material, which was removed with a pipette, and the tissues were then placed in a weighed Erlenmeyer flask. In the case of mussels, a much smaller number of animals was used. The relatively large amount of sea water within the mantle cavity of the mussel was drained off and discarded before removing the soft parts.

The wet weight of the soft tissues of barnacles and mussels was obtained and the approximate volume of the wet tissue was determined by forcing it into a graduated cylinder.[3] The material was transferred quantitatively to a flask, dried in an oven at 100° C., and the dry weight determined. Enough H_2SO_4 was added to char the material. It was heated to fumes on a hot plate, and concentrated HNO_3 was added dropwise until a colorless solution was obtained. The solution in the flask was transferred quantitatively to a 100 cc. volumetric flask and diluted to the mark. Test for copper was made by the carbamate method (Coulson, 1937). A blank to which the same amount of reagent was added was also carried through the above procedures. In the analysis of barnacle shells, the wet weight of the shells was first

[3] In the early experiments, volume in cubic centimeters alone was determined, but was subsequently found to be very nearly equal to the wet weight in grams.

determined. The shells were then dried in the oven at 100° C. overnight and the dry weight determined. The material was ignited in the muffle furnace at 800°–900° C. for three hours, removed and cooled. Two cubic centimeters of concentrated HCl were added, warming if necessary to dissolve.[4] The remainder of the analysis followed the same procedure as with the soft tissues.

Determinations revealed that the amount of copper in the shell tissue of both normal and poisoned barnacles was quite variable. There was also no consistent change in the copper content of the shells in the case of batches of poisoned barnacles with increasing amounts of copper in the soft tissues. Accordingly, in the later experiments the laborious and rather unsatisfactory copper assay of the shells was omitted.

The copper content of barnacles freshly brought into the laboratory is many times greater than an equal volume or an equal weight of sea water (Table V). Evidently under natural conditions, the barnacle tends to accumulate copper in its tissues to a considerable extent.

TABLE V

Copper content of freshly collected barnacles
Woods Hole sea water contains 0.00001 to 0.00003 mg./cc.

Species	Source	Number	Copper in soft parts			Copper in shells		
			mg.	mg./cc.*		mg.	mg./cc.	
Balanus eburneus	Cotuit, July 28	50	0.0017	0.0014		0.141	0.027	
	Cotuit, July 28	50	0.0027	0.0024		0.097	0.014	
			mg.	mg./g. Wet wt.	mg./g. Dry wt.	mg.	mg./g. Wet wt.	mg./g. Dry wt.
Balanus eburneus	Cotuit, Aug. 20	50	0.015	0.0038	0.008	0.007	0.0003	0.0004
	Cotuit, Aug. 20	40	0.010	0.0019	0.014	—	—	—
	Cotuit, Sept. 12	30	0.016	0.0011	0.016	—	—	—
Balanus balanoides	Woods Hole, July	100	0.029	0.029	0.104	0.069	0.0070	0.0075
	Buzzards Bay	50	0.023	0.027	0.068	0.063	0.0065	0.0067

* May be assumed equivalent to mg./g. wet weight.

In experiments designed to test whether barnacles could accumulate copper in their tissues, it was found that the amount of copper present increased rapidly for each day that the animals remained in the toxic solution. At the end of four days of continuous poisoning in the two concentrations used, all the animals were dead (Blocks A and C, Tables VI and VII). However, in those cases in which the barnacles were transferred to fresh sea water after one or two days, the animals were restored to what appeared to be a healthy condition.[5] Analysis of the tissues of the barnacles which had been replaced in fresh sea water showed that the animals had not only recovered their activity but also had eliminated a certain amount of copper. These barnacles still contained, however, as much as twenty-five times the amount of copper found in their tissues before poisoning began.

[4] If an excess of $CaCO_3$ remained, more acid was added until evolution of CO_2 ceased.

[5] The recovery of Bugula larvae from copper poisoning was reported by Miller and Cupp (1942) and Miller (1946).

TABLE VI

Changes in copper content of soft tissues during poisoning and recovery

Toxic: Cupric citrate, conc. 0.20 mg. Cu/liter
Test Animal: Balanus eburneus
Date: August 3–8, 1942
*Basis: Copper content per cubic centimeter of soft tissue **

Block A		Block B	
Initial copper content	0.010 mg.	Initial copper content	0.010 mg.
After 1 day in toxic	0.034 mg.	After 1 day in toxic	0.034 mg.
After 2 days in toxic	0.070 mg.	After 1 day in sea water	0.033 mg.
After 3 days in toxic	0.093 mg.	After 2 days in sea water	0.030 mg.
After 4 days in toxic	0.112 mg.	After 3 days in sea water	0.035 mg.
		After 4 days in sea water	0.026 mg.
Condition at end—dead		Condition at end—open, inactive but reacts to touch	

* May be assumed equivalent to wet weight.

TABLE VII

Changes in copper content of soft tissues after varying exposures to toxic and after recovery

Toxic: Cupric citrate, conc. 0.35 mg. Cu/liter
Test Animal: Balanus eburneus
Date: August 10–17, 1942
*Basis: Copper content per cubic centimeter of soft tissue **

Block C		Block D		Block E	
Initial Cu content	0.002 mg.	Initial Cu content	0.002 mg.	Initial Cu content	0.002 mg.
After 1 day in toxic	0.024 mg.	After 1 day in toxic	0.024 mg.	After 1 day in toxic	0.024 mg.
After 2 days in toxic	0.073 mg.	After 2 days in toxic	0.073 mg.	After 1 day in sea water	0.029 mg.
After 3 days in toxic	0.089 mg.	After 1 day in sea water	0.056 mg.		
After 4 days in toxic	0.075 mg.	After 9 days in sea water	0.054 mg.	After 6 days in sea water	0.022 mg.
Condition at end—dead.		Condition at end—cirri give incomplete sweep or full sweep but slow.		Condition at end—normal rapid sweep of cirri.	

* May be assumed equivalent to wet weight.

These indications that barnacles can take up relatively large quantities of copper without being killed and can eliminate the poison to a certain extent were confirmed by more elaborate experiments which were designed primarily to test the effects of the toxic and of different periods of exposure and of recovery (Tables VIII to XI).[6] These experiments demonstrated that barnacles took up copper

[6] In these experiments the dry weight of the soft tissues to be analyzed was determined and the copper content stated on the more accurate basis of milligrams of copper per gram of dry weight. The dry weight for the soft parts of the barnacle was found to be about 5 per cent to 20 per cent of the wet weight. Since this group of experiments was conducted during late summer and autumn, there exists the possibility that the results may not be strictly comparable with the earlier work. The barnacles tested were necessarily older and larger, and were no doubt in a different physiological condition. It was noted that the animals became less active as the season progressed, and by late autumn they tended to remain closed for long periods. This last fact alone would be expected to cause an important difference in the rate at which toxic was absorbed. In general, the animals tended to survive longer in high concentrations of toxic in the autumn than they had in the summer.

TABLE VIII

Comparison of changes in copper content of soft tissues during continuous exposure to low concentration of toxic and intermittent exposure to higher concentration

Toxic: Cupric citrate, Block F: 0.14 mg. Cu/l.; Block G: 0.22 mg. Cu/l.
Test Animal: Balanus eburneus
Date: August 24 to September 28, 1942
Basis: Copper content per gram of dry weight of soft tissue
Exposure times indicated for toxic are cumulative totals

Block F		Block G	
Initial copper content	0.014 mg.	Initial copper content	0.008 mg.
After 1 day in toxic	0.05 mg.	After 1 day in toxic	0.037 mg.
After 2 days in toxic	0.08 mg.	(Replaced in sea water for 1 day)	—
After 7 days in toxic	0.33 mg.	After 2 days in toxic	0.095 mg.
After 15 days in toxic	1.09 mg.	(Replaced in sea water for 1 day)	—
(Replaced in fresh sea water)		After 3 days in toxic	0.18 mg.
After 18 days in sea water	0.39 mg.	(Replaced in sea water for 6 days)	—
		After 4 days in toxic	0.23 mg.
			(0.19 mg.)*
		(Replaced in sea water for 7 days)	0.17 mg.
		After 7 days in toxic	0.48 mg.
		(Replaced in sea water for 16 days)	0.51 mg.
Condition at end—full but slow sweep of cirri		Condition at end—full but slow sweep of cirri	

* Batch of dead barnacles.

faster from the more concentrated toxic media. With the slower rate of absorption at the lower concentrations, the animals appeared to withstand higher amounts of copper in their tissues. Thus the highest copper content observed for living barnacles, namely, 1.09 mg. per g. dry weight, was found after fifteen days in 0.14 mg. Cu/liter cupric citrate (Table VIII, Block F). In cases in which large amounts of copper were absorbed the barnacles tended to live longer and to remain in better condition when periods in sea water were alternated with periods in the toxic medium.

In cases where the copper content was determined for the dead barnacles, which were found among the living individuals in increasing number as the experiment progressed, it was almost invariably true that the amount of copper present was less than that for the living specimens from the same block. This observation suggests that the entrance of copper and its fixation in the tissues of the barnacle is not simply a mechanical process of diffusion or adsorption but is a process which is influenced in some way by the metabolic reactions of the living organisms.

Due to the facts considered above, it is not possible to arrive at any definite value for an absolute amount of copper which must be absorbed by the barnacle to kill it, nor at any rate of absorption above which death will ensue. The results suggest that killing may not be due to the destruction of some vital substance or process by the direct action of the copper as much as to a general depressing action of the toxic on some function, such as the feeding reaction, to the extent that death eventually follows.

TABLE IX

Comparison of changes in copper content of soft tissues during continuous and intermittent exposures to high concentration of toxic and continuous exposure to lower concentration

Toxic: Block H—Cupric citrate 0.75 mg. Cu/l. Continuous
Block I—Cupric citrate 0.35 mg. Cu/l. Continuous
Block J—Cupric citrate 0.75 mg. Cu/l. Alternating daily with sea water
Test Animal: Balanus eburneus
Date: September 21 to October 14, 1942
Basis: Copper content per gram of dry weight of soft tissue

Block H		Block I	
Initial Cu content	0.016 mg.	Initial Cu content	0.016 mg.
After 2 days in toxic	0.17 mg.	After 3 days in toxic	0.13 mg.
After 4 days in toxic	0.30 mg.	After 6 days in toxic	0.25 mg.
After 6 days in toxic	0.38 mg.	After 9 days in toxic	0.32 mg.
After 7 days in toxic	(0.33 mg.)*	After 11 days in toxic	0.76 mg.
After 8 days in toxic	0.48 mg.	After 14 days in toxic	(0.39 mg.)*
After 11 days in toxic	(0.84 mg.)*	After 14 days in toxic	0.49 mg.
		After 18 days in toxic	(0.43 mg.)*
		After 18 days in toxic	0.60 mg.

Block J

Initial Cu content		0.016 mg.
After 2 days in toxic,	1 day in sea water	0.16 mg.
After 4 days in toxic,	3 days in sea water	0.13 mg.
After 6 days in toxic,	6 days in sea water	0.31 mg.
After 8 days in toxic,	7 days in sea water	0.42 mg.
After 8 days in toxic,	8 days in sea water	0.25 mg.
After 10 days in toxic,	9 days in sea water	0.48 mg.
After 12 days in toxic,	11 days in sea water	0.50 mg.
After 12 days in toxic,	11 days in sea water	(0.48 mg.)*

* Batch of dead barnacles.

TABLE X

Changes in copper content of soft tissues during recovery from various exposures to toxic

Toxic: Cupric citrate 0.75 mg. Cu/l.
Test Animal: B. eburneus
Date: October 21–November 12, 1942
Basis: Copper content per gram of dry weight of soft tissue

	Treatment	Copper content	Condition
Block L	After 3 days in toxic	0.38 mg.	++
	After 10 days in sea water	0.31 mg.	+
	After 14 days in sea water	0.21 mg.	+
Block M	After 6 days in toxic	0.38 mg.	+
	After 7 days in sea water	0.36 mg.	+
	After 14 days in sea water	0.40 mg.	++
Block N	After 9 days in toxic	0.58 mg.	0+
	After 9 days in toxic	(0.39 mg.)	dead individuals
	After 7 days in sea water	0.43 mg.	+
	After 14 days in sea water	0.39 mg.	++

No barnacles were found to have been killed by the absorption of less than 0.19 mg. of copper per g. of dry weight—an amount which is ten or more times the normal content of the tissues. In many cases animals succumbed after about 0.4 mg. per gram of dry weight had been taken up by the tissues. In no case did the barnacles absorb more than 1.09 mg. of copper per gram of dry weight. Barnacles which had accumulated copper from toxic solutions to the extent of 0.5 mg. to 1.09 mg./g. dry weight, in some cases revived when replaced in fresh sea water and were still alive two or three weeks later. At the end of the period, when they had regained normal, or nearly normal, activity, they contained 0.3 to 0.5 mg. of copper per g. of dry

TABLE XI

Changes in copper content of soft tissues during recovery from exposures at high concentrations of toxic

Toxic: Block O—Cupric citrate 0.90 mg. Cu/l.
Block Q—Cupric citrate 0.75 mg. Cu/l.
Test Animal: B. eburnesus
Date: October 21–November 21, 1942
Basis: Copper content per gram of dry weight of soft tissue

	Treatment	Copper content	Condition
Block O	After 8 days in toxic	0.52 mg.	0+
	After 8 days in toxic	(0.48 mg.)	dead individuals
	After 8 days in sea water	0.42 mg.	0+
Block Q	After 9 days in toxic	0.53 mg.	0+
	After 9 days in toxic	(0.35 mg.)	dead individuals
	After 8 days in sea water	0.40 mg.	+
	After 15 days in sea water	0.35 mg.	++

weight. In most instances the barnacles eliminated copper from their tissues, especially in cases where a high concentration had been accumulated. Although the copper which had been absorbed was never entirely eliminated, and although the process was slower than for accumulation, the experiments show that barnacles have some ability to rid their tissues of copper.

Accumulation of copper in mussel tissue during poisoning and recovery

To determine the amount of copper taken up by the tissues of the mussel when subjected to copper solutions, groups of 25 mussels were placed in various concentrations of cupric citrate and were later transferred to fresh sea water after increasing periods of time. The media were renewed every day. The copper content of 20 fresh animals from the same source was assayed at the beginning of the experiment and batches of three to five animals were withdrawn from the various groups for copper assay after the indicated number of days during poisoning and during recovery (Table XII).

The initial copper content of the mussel tissue was 0.0109 mg. per gram of dry weight, roughly the same as for the barnacle. Since the wet weight of the soft parts of the mussel was similarly found to be about ten times the dry weight, it is

clear that the fresh mussel also contains many times the concentration of copper present in the sea water.

After the first day in the toxic media, the mussels in the more concentrated solutions had taken up six to eight times their original amount of copper, but in the weaker solutions, there was no consistent increase in copper content until after an exposure of three days. The group of mussels (Group 4) in the weakest solution had absorbed only about two and one-half times their initial copper content after five days—or less than half the amount the Group 1 mussels had absorbed in one day in a solution five times as strong. In addition, the mussels were found to be able to rid their tissues of copper effectively. One day's sojourn in fresh sea water was suffi-

TABLE XII

Changes in copper content of soft tissues of mussel during poisoning and recovery

Toxic: Cupric citrate
Test Animal: Mytilus edulis, 25 animals (5 cm. long) in each group
Date: August 19 to September 5, 1942
Basis: Copper content in milligrams per gram of dry weight of soft tissue

Exposure to toxic solution began August 19. The line marks the time when mussels were transferred to fresh sea water

Group	Concentration of Cu citrate mg. Cu/l.	No. of days in toxic	Content on indicated date							
			Aug. 20	Aug. 21	Aug. 22	Aug. 23	Aug. 24	Aug. 25	Aug. 26	Sept. 5
1	0.120	1	0.066	0.0196	—	—	—	—*	—	—
2a	0.082	1	—	0.0135	—	—	0.017	—	—	0.0085
2b	0.082	2	0.084	0.031	0.023	—	—	—	—	0.0077
3	0.049	3	0.0076	—	0.024	0.018	—	—	0.017	0.0100
4	0.027	5	0.0135	—	0.021	—	0.025	0.029	—	0.0130
Control	0	0	0.0109							

* Condition on August 25: Group 1—all dead
Group 2a—9 dead
Group 2b—10 dead
Group 3—3 dead
Group 4—4 dead

No further deaths to September 5.

cient to reduce the amount of copper in the mussels from the strongest solutions by more than 70 per cent. In cases where longer exposures were employed (although in lower concentrations) more time seemed to be required for the elimination of the copper—perhaps because it had penetrated to deeper tissues. At the end of about two weeks in sea water, the copper content of the mussel tissue was back to normal—a fact quite in contrast to the situation with the barnacle.

In spite of the fact that after one day in sea water the copper content of the Group 1 mussels had been reduced at least to the amount reached by the mussels in the weaker solutions, all the animals in this group died within five days. Some irreparable damage had been done by the single day's exposure to the high concentration of the toxic. In the lower concentrations only three to four animals died

despite exposures to the toxic for three to five times as long. It is possible that the mussels in the lower concentrations could eliminate copper from vital tissues as fast as it tended to enter.

Although the values obtained in this single experiment are somewhat irregular, two important differences are indicated by the results as compared with the results obtained with barnacles. At the same concentrations, the exposures which are lethal to mussels are considerably shorter than for barnacles. Similarly the mussels succumbed after accumulating amounts of copper in their tissues which were much lower than was the case with barnacles.

Tests on the Poisoning of Cyprids

During January, 1943, experiments were undertaken at Miami Beach, Florida, on the poisoning of barnacles in the cyprid stage. Especial interest centered on these observations because the cyprid is the stage in which the animal first attaches itself to the substratum. It is the opinion of many that once a barnacle becomes attached, it can be killed only with great difficulty and that even if it is killed, the shell does not drop off but remains firmly attached. Actually, attachment takes place in two steps: first, the attachment of the antennae of the cyprid, following which metamorphosis is begun; and second, the attachment of the base of the barnacle after metamorphosis has been completed.

Cyprids were obtained by suspending glass microscope slides in Biscayne Bay overnight. The cyprids were presumed to be those of *Balanus improvisus*, which is the commonest adult barnacle in the neighborhood. The slides were brought into the laboratory where the following details in the development of the cyprids were observed.

The cyprids are originally attached by their antennae. About 5 or 6 hours after attachment, the animal undergoes metamorphosis in the course of which the cyprid shell is moulted and the animal emerges as a more or less round body still adhering to the substratum by the original attachment which now appears as the center of the convex base of the animal.

As development proceeds the base of the animal, which was at first freely movable, tends to flatten and to be pressed against the substratum. About 24 hours after metamorphosis the base is completely flattened with relatively sharp edges. A pulsation of the central parts of the animal has begun by this time, and the operculum may open, but no cirri are extended. The base is not caused to move by the activity of the animal, as before, but any part of it, except the original central point of attachment, is easily dislodged by pressure with a needle.

After another 24 hours, the cirri may be extended and filter actively, and the main area of the base begins to adhere to the substratum. At first the base merely gives the impression of being sticky; later it becomes attached with sufficient firmness so that it cannot be dislodged without tearing the tissue. Not until the third day or later does hard material (presumably calcium carbonate) begin to appear in the base.

The attachment of the cyprid by its antennae takes place quickly, and the original attaching structure remains in existence at least until calcification begins. The attachment of the base of the newly metamorphosed barnacle is a slow process which provides only weak adhesion at first but which finally supplies a permanent and

TABLE XIII

Effect of different concentrations of cupric citrate in preventing the metamorphosis of barnacle cyprids attached to glass slides

Species: probably Balanus improvisus

Concentration mg. Cu/liter	Number of cyprids exposed	Number of cyprids which metamorphosed
116.	6	2 (moult incomplete)
58.	6	3 (moult incomplete)
23.	4	4
9.7	3	3
4.9	2	2
0.93	11	11
0.47	9	9
0.23	5	5
0.12	9	9

firmly cemented calcareous structure. Poisoning of the barnacle could theoretically be accomplished either in the attached cyprid stage or as a newly metamorphosed animal before the calcification of the base.

The susceptibility of the cyprid to poisoning was investigated by placing cyprids attached to glass slides in solutions of cupric citrate (Table XIII) and of mercuric chloride (Table XIV). In the case of the copper solutions, the cyprids metamorphosed successfully in concentrations as great as 23 mg. Cu/l and solutions as strong as 116 mg. Cu/l were only partially inhibitory. Concentrations about 100 times greater than those ordinarily tolerated by adult barnacles, therefore, were not capable of killing the cyprids before they completed their metamorphosis.

In the case of the mercury solutions, although an inhibitory effect was observed at a lower concentration (16.6 mg. Hg/liter) than for copper, much higher concentrations of the toxic were required to prevent metamorphosis than would ordinarily kill adult barnacles.

Failure to kill the cyprid may be due to an unusually high resistance of the animal to poison in this stage or to the relative shortness of the period between attachment and metamorphosis, or both. From these results it is probable that the metamorphosis of the attached cyprid cannot be stopped by any concentration of toxic which could practically be obtained from a paint.

TABLE XIV

Effect of different concentration of mercuric chloride in preventing the metamorphosis of barnacle cyprids attached to glass slides

Species: probably Balanus improvisus

Toxic solution mg. Hg/liter	Number of cyprids exposed	Number which metamorphosed
83.	5	0
16.6	6	3 (moult incomplete)
3.3	6	6
0.66	9	9
0.17	7	7
0.09	10	10

The susceptibility to poisoning of the newly metamorphosed barnacle was tested in a similar fashion by placing slides bearing animals in this stage in solutions of cupric citrate (Table XV). Much lower concentrations of copper were required to kill the newly metamorphosed barnacle than was necessary to prevent the cyprids from carrying out their metamorphosis. The relation between killing time and concentration is roughly of the same order of magnitude as observed for adult barnacles at Woods Hole. There is some indication, however, that the newly metamorphosed animals are slightly more resistant, as concentrations of 0.23 mg. Cu/1 to 0.47 mg. Cu/1 were required to kill in five days in contrast to concentrations of only 0.14 to 0.30 mg. Cu/1 for the older animals.

Although recently metamorphosed barnacles survived for several days in concentrations of cupric citrate of 0.12 mg. Cu/1 or lower, their development was very materially retarded. In the stronger solutions the base remained in the rounded

TABLE XV

Relation of killing time to concentration of cupric citrate in barnacles which had just completed metamorphosis

Species: probably Balanus improvisus

Concentration mg. Cu/liter	Killing time days
9.7	1
4.9	1–2
0.97	3
0.93	3–4
0.47	5
0.23	5
0.20	7
0.12	10

condition, and in concentrations down to at least 0.20 mg. Cu/1 the base never became calcified nor rigidly cemented to the substratum, although it might become flattened and adhesive.

In a small number of tests reported subsequent to this investigation by Pyefinch and Mott (1944), free swimming cyprids of *Balanus balanoides* were killed in 24 hours by 0.5 to 1.0 p.p.m. copper from cupric sulphate. Metamorphosis was completed in concentrations up to 7 p.p.m. but after metamorphosis the barnacles were killed in 3 days by 0.5 p.p.m. Mercury from mercuric chloride was found to be more toxic than copper. These results agree very satisfactorily with ours.

At still lower concentrations of copper, in the present experiments, a complete change in the effect of the metal evidently comes about, for development was accelerated. Animals in the lowest concentration tested, namely 0.06 mg. Cu/1, reached the stage of active filtering with their cirri in less than 48 hours after metamorphosis, although the controls did not attain this stage until about 2 days later. It appears that a small amount of copper acted as a stimulant to development, and that larger amounts of copper retarded development even though they may be insufficient to kill the animals outright.

Discussion

Adult barnacles, and perhaps young stages also, can absorb ten to possibly one hundred times as much copper as their tissues normally contain without apparent injury. They can eliminate a certain amount of this material also. Therefore, when copper is used as a toxic in an antifouling paint, a substance is employed which can be taken up with impunity in relatively large quantities by the barnacle and to a lesser extent by the mussel. The animals can recover from the effects of this poison, to a certain extent at least, when again bathed by fresh sea water.

In some cases mussels which had received a lethal dose of poison remained in an apparent healthy condition for several days after being replaced in fresh sea water before dying. No such latent period was observed in the experiments with barnacles.

Possibly related to the facts just considered is the indication that the action of copper as a poison is not so much the direct destruction of some tissue or vital material in the barnacle as a general retardation of life processes. In the case of the adult barnacle, subjection to copper solutions causes a slowing and eventual cessation of the filtering activity of the cirri. In the case of the newly metamorphosed stages, not only is movement inhibited but development is seriously retarded as well. Much more satisfactory as a poison would be some substance which struck directly and irreversibly at some vital point in the animal and which could not be eliminated from the tissues. With such an ideal poison, barnacles which received only small doses, due to unavoidable dilution, would eventually be killed when a lethal amount of the material had accumulated. We have shown that with copper, sublethal doses may possibly stimulate development and certainly may allow subsequent recovery. Evidence that copper may stimulate the attachment of larvae has been reported by Prytherch (1934) for the oyster and by Miller (1946) for *Bugula*.

The relative vulnerability of the various steps in the attachment and growth of barnacles has been revealed by this investigation. The original idea that barnacles could be eliminated from a ship's hull only by preventing their initial attachment is partly right and partly wrong. As explained above, the attachment actually takes place in two steps. There is little promise of using poison successfully to prevent the first step, namely the attachment of the cyprid by its antennae, since this apparently takes place in a matter of minutes. However, we should not lose sight of the very important, but as yet unrealized, possibility of finding a substratum toward which the cyprid would display an avoiding reaction or to which the cement of the antennae would not adhere.[7]

Our tests have demonstrated the practical impossibility of preventing by poison the metamorphosis of the cyprid or of killing it during the process. This conclusion is substantiated by the results of Pyefinch and Mott (1944) and is in agreement with their statement: "Settlement and metamorphosis can take place on a paint in other respects anti-fouling though death of the barnacle occurs after metamorphosis." Similarly, at the other end of the life cycle, after the adult barnacle has attained any considerable size, it can be poisoned only with great difficulty for the reasons already discussed.

[7] In certain tests under laboratory conditions, Pyefinch and Mott (1944) found that cyprids failed to settle in concentrations of copper as low as 0.03 p.p.m.

There remains, however, the possibility of effectively preventing the second step in the attachment process, namely the permanent cementing of the base of the newly metamorphosed animal to the substratum. Although a long exposure to copper or a high concentration is necessary to kill the barnacle outright at this stage, moderate concentrations of poison tend to retard development and possibly to prevent the formation of the calcareous base, or the cement for this base, with the result that the young barnacle never succeeds in establishing a firm attachment, and hence is eventually displaced. The newly metamorphosed stage, therefore, appears to be the most vulnerable to copper poisoning.

The indication is, then, that copper paints act by preventing attachment but do so chiefly during the second step of the process through interference with the final cementing of the base of the metamorphosed barnacle to its substratum.

Summary

1. Direct tests were performed on the concentrations and exposures of a variety of metallic salts necessary to kill barnacles. The toxicities of mercury, cupric citrate, cupric tartrate, cupric salicylate, and cupric aminobenzoate were found to be slightly less than the toxicity of basic cupric carbonate. The toxicity of silver is about equal to that of basic cupric carbonate, but the toxicity of zinc is very much less.

2. The rate of killing of barnacles by cupric citrate is proportional to the concentration of the toxic over the range tested.

3. An extremely high concentration of copper or of mercury salts was necessary to prevent the metamorphosis of cyprids attached to glass plates. The results show the difficulty of preventing the *initial* attachment of cyprids, or their metamorphosis, by the use of copper paints.

4. Moderate concentrations of cupric citrate seriously retard the development of the newly metamorphosed barnacles and prevent the second step in attachment, namely, the formation of the cemented calcareous base.

5. Exposure of the newly metamorphosed barnacles to very low concentrations of cupric citrate accelerated development beyond that of the normal animals.

6. The soft tissues of adult barnacles normally contain a much higher concentration of copper than does sea water. When placed in solutions of cupric citrate, barnacles absorbed more than 100 times the normal copper content of the tissues. In no case were barnacles killed by the absorption of less than 0.19 mg. of copper per gram of dry weight—more than 10 times the normal content. In some cases barnacles which had absorbed 0.5 mg. to 1.09 mg./g. from toxic solutions revived when replaced in fresh sea water.

7. It was demonstrated that when replaced in fresh sea water, barnacles can eliminate from their tissues as much as two-thirds of the copper which has been absorbed from toxic solutions.

8. Mussels are more sensitive to poisoning by cupric citrate than barnacles. When exposed to copper solutions, mussels take up copper more rapidly than do barnacles, and when replaced in fresh sea water, they eliminate it from their tissues more rapidly and extensively. In many cases in which a considerable portion of the copper was eliminated, the mussels nevertheless succumbed subsequently.

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EFFECTS OF HYDROGEN PEROXIDE PRODUCED IN THE MEDIUM BY RADIATION ON SPERMATOZOA OF ARBACIA PUNCTULATA

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In the course of a previous investigation (Evans et al., 1941, 1942a) it was found that roentgen radiation exerted effects on Arbacia sperm indirectly by producing a very temporary toxicity of the medium. The intermediary appeared to be the "activated water molecule," as postulated by Fricke (1935), and was characterized by the following phenomena. Sperm irradiated in sea water suffered immediate reduction in fertilizing capacity, whereas sperm irradiated "dry" were apparently unaffected in this respect. The smaller the concentration of sperm in the suspension during irradiation the greater the percentage made incapable of fertilization. Presence of proteins, such as egg albumin, afforded "protection," that is, greatly increased the percentage of sperm surviving irradiation. Unirradiated sperm did not suffer immediate reduction in fertilizing capacity when placed in water that had been exposed to amounts of radiation capable of producing such effects when the sperm were present during the irradiation. Under certain conditions "activated water" reacts to produce hydrogen peroxide (Risse, 1930; Taylor et al., 1933; and Fricke, 1934a) which is toxic to organisms and may account for some or all of the indirect effects of X-rays. To understand better the indirect action of X-rays on cells, comparative studies of the effects of irradiated water and hydrogen peroxide were made on Arbacia sperm and ova.

Materials and Methods

Male Arbacia were stimulated, by cutting through the oral region, to shed sperm in a dish of sea water. The suspension was centrifuged until the sperm were closely packed, then the supernatant fluid was removed. Before placing the sperm in test solutions, nine parts of sea water were mixed with one part of packed sperm in order to facilitate transfer of equal amounts of sperm suspension to the various dishes. One part of this suspension was added to ninety-nine parts of the test solution making a final concentration of 1 : 1,000 sperm. Percentage fertilizing capacity was determined by inseminating a given lot of freshly collected eggs with as many drops of control sperm as needed to produce 90–99 per cent of the eggs with

[1] It is a pleasure to express appreciation to Dr. G. Failla for his helpful criticisms and to Miss Anne R. Behan for technical assistance.

fertilization membranes. The same volume of sperm from the experimental medium was then tested with a lot of eggs similar to those used for the controls.

Samples of sperm from sea water and from experimental media were taken at regular intervals and tested for fertilizing capacity. The survival period was taken as the time required to reduce the percentage fertility of a given lot of sperm to less than 50 per cent. The actual survival time was somewhat longer in each case as some sperm would still be alive after the suspension had completely lost the ability to fertilize untreated eggs. A few observations were made of longevity as indicated by rate of oxygen consumption and motility of sperm. The relative decrease in survival time of different experimental lots was approximately the same whether these criteria or fertilizing capacity were employed. Sperm in a concentration of 1 : 10 suffered no change in fertilizing capacity during the time covered by these experiments. Therefore, when it became necessary to employ a new batch of eggs, the proper number of drops of sperm suspension to use in each test was determined by employing a freshly diluted 1 : 1,000 suspension. Cleavage time was taken as the number of minutes (at 25° C.) from the time of insemination until 50 per cent of the fertilized eggs had undergone division. Catalase solutions used were sea water dilutions of a stock extracted from sperm by distilled water. Albumin solutions were 0.1 gram per cent of powdered egg albumin made up in sea water and used immediately.

Fresh filtered sea water was irradiated in covered plastic dishes at 22–28° C. The material was cross-fired (from above and below) between two water-cooled X-ray tubes operated at 182 kv pk., and 25 ma (inherent filtration equivalent to approximately 0.2 mm. Cu). The intensity was usually 5,600 r per minute and the irradiation was given in one treatment.

Experimental Results

Effects of irradiated water

Results of exploratory experiments (Evans et al., 1942a; Evans, 1942) indicated that irradiated water reduced the survival time and prolonged the cleavage time of sperm placed therein. The amount of effect varied greatly from one lot of sperm to another. It appeared in general that irradiation below 50,000 r was ineffectual, around 100,000 r was definitely effective, and a dose of 224,000 r was near the optimum in affecting the water so that it reduced survival time of sperm. The delay in first cleavage (treated sperm + untreated ova) was noticeable when only low dosages (below 100,000 r) had been given to the water, but the effect did not appear to increase rapidly as the irradiation became greater. In the exploratory experiments chemical tests indicated presence of hydrogen peroxide in the heavily irradiated water, and this agent was also found to affect survival time and cleavage time of sperm. It was now of interest to determine quantitatively how much of the toxicity of the irradiated water should be attributed to hydrogen peroxide.

It was found that consistent results were obtained when sperm from several males were pooled so that successive experiments were made from the same original collection. The data are shown in Figure 1 and Figure 2. It may be seen in Figure 1 (top graph) that, in all four experiments, the water became more toxic as the amount of radiation was increased. Reduction in survival time by doses below

85,000 r was not very definite. The effect increased rapidly from 85,000 r to 140,000 r and increased, though less rapidly, from 140,000 r to 230,000 r. The latter finding may indicate that equilibrium was being reached between the rate of hydrogen peroxide formation and the rate of destruction by the radiation.

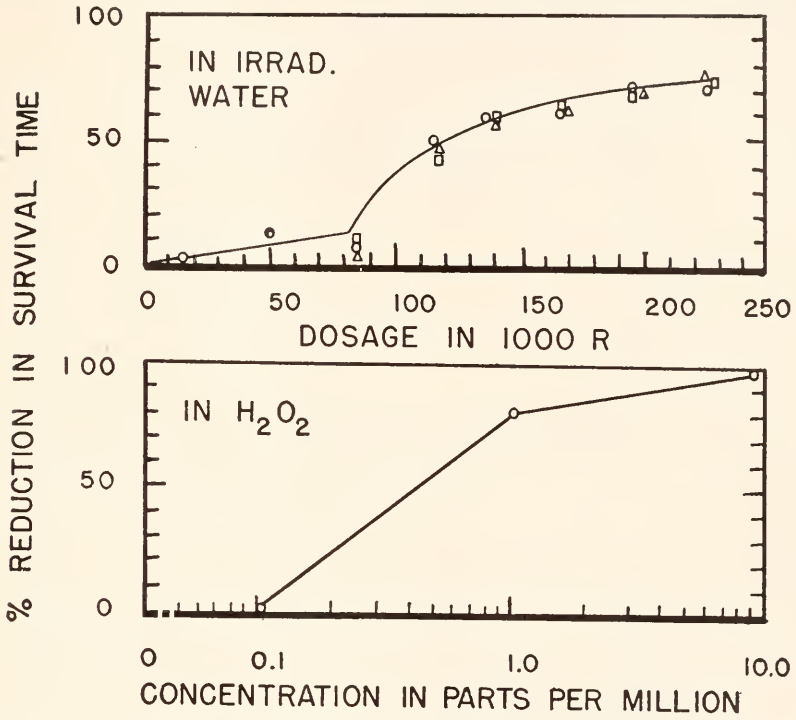

FIGURE 1. Upper graph: effect of different amounts of radiation on the toxicity of sea water to sperm. Toxicity is indicated by a reduction of the survival time (as measured by fertilizing capacity) of the sperm placed in the irradiated water as compared to the survival time of sperm in control sea water.

Lower graph: toxicity of different amounts of hydrogen peroxide in sea water to sperm placed therein.

The effect of retarding cleavage was greater as the irradiation of the water was increased (upper graph of Fig. 2). Also, the cleavage time was more delayed the longer the sperm had been in the irradiated water at the time of insemination. This is shown in the lower graph of Figure 2. The effect approached a maximum, at this concentration of the agent, in about an hour (at 25° C.).

Effects of hydrogen peroxide

Calculations based on data available in the literature (Fricke, 1934a, 1934b; Taylor, Thomas, and Brown, 1933) and color tests, using titanic chloride (Evans et al., 1942a), of the irradiated sea water indicated that we were dealing with concentrations of hydrogen peroxide in the neighborhood of 1 : 1,000,000. The effects of different concentrations of hydrogen peroxide [2] on the survival time are shown

[2] Merck's Superoxol reagent hydrogen peroxide (30 per cent).

in the lower graph of Figure 1. One would judge from a consideration of the two graphs of Figure 1 that the maximum amount of hydrogen peroxide produced in the irradiated water was approximately 1 : 1,000,000.

Hydrogen peroxide in a concentration of 1 : 1,000,000 retarded cleavage (lower graph of Fig. 2) and the increase in effect with longer exposures was very similar to that of sea water given an irradiation of 224,000 r.

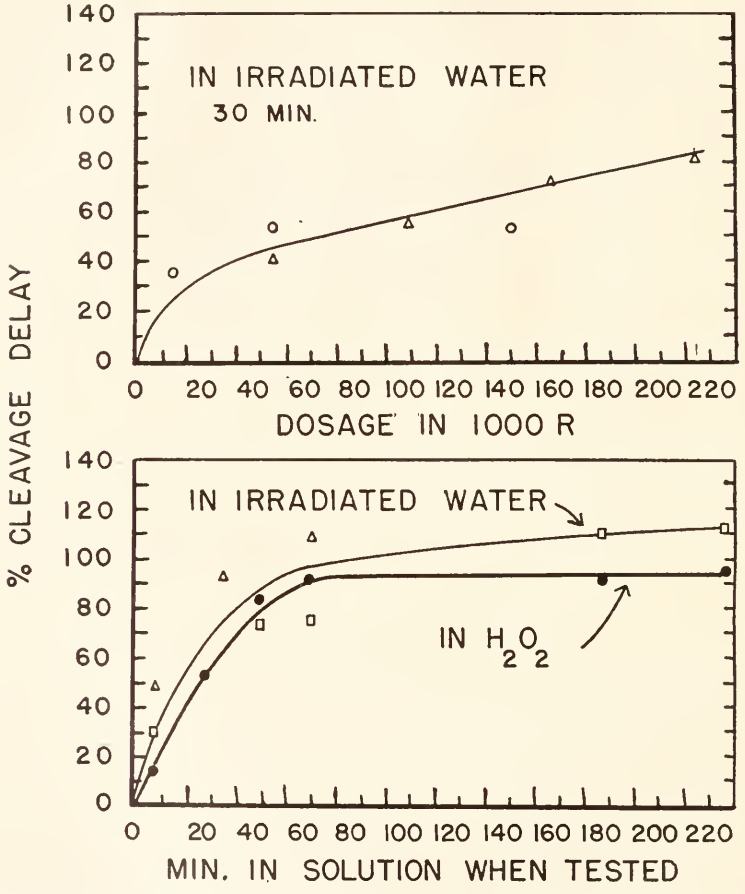
% CLEAVAGE DELAY
IN IRRADIATED WATER
30 MIN.
DOSAGE IN 1000 R
IN IRRADIATED WATER
IN H_2O_2
MIN. IN SOLUTION WHEN TESTED
0 20 40 60 80 100 120 140 160 180 200 220
0 20 40 60 80 100 120 140

FIGURE 2. Upper graph: effect of different amounts of radiation on toxicity of sea water as indicated by a retardation in time of first cleavage. After sperm had been in the irradiated water for 30 minutes they were used to inseminate fertile eggs in fresh sea water. Cleavage delay is expressed as cleavage time over that of eggs inseminated with control sperm.

Lower graph: effects (on cleavage time) of exposing the sperm for different periods of time to irradiated water (224,000 r) and to water containing hydrogen peroxide (1 : 1,000,000).

Reduction in toxicity of irradiated water by catalase extract

't was found that a crude extract possessing catalase activity could be removed concentrated sperm suspensions.[3] This extract could be added to sperm in

[3] Report in preparation.

sea water without producing any apparent deleterious effects. Results of adding catalase to irradiated water are shown in Tables I and II. It will be seen that the catalase extract completely removed the toxic agent responsible for decrease in survival time (Table I) in all but three instances and greatly reduced the effect in these.

TABLE I

REDUCTION OF TOXICITY OF IRRADIATED WATER BY CATALASE EXTRACT

*(Percentage decrease in survival time as compared to sperm in control sea water)**

In irrad. water (160,000 r)	In irrad. water +inactive catalase**	In irrad. water +active catalase	In H_2O_2 (1:1,000,000)	In H_2O_2 +active catalase
50	50	8	—	—
92	—	0	90	0
55	55	0	—	—
38	38	0	—	—
81	43	22	—	—
83	48	0	—	—
50	—	0	94	0
81	—	57	—	—

* There were three control groups (1) sperm in untreated sea water, (2) sperm in sea water plus inactive catalase, and (3) sperm in sea water plus active catalase. There was no decrease in survival time of groups 2 and 3 as compared to group 1. Control time was taken as that of group 1.

** Inactivated by heating (60–65° C.) for 30 minutes.

TABLE II

REDUCTION IN TOXICITY OF IRRADIATED WATER BY CATALASE EXTRACT

(Cleavage delay, as compared to controls, in percentage)

Time in experimental medium	In irrad.* water	In irrad. water +inactive catalase	In irrad. water +active catalase	In H_2O_2 (1:1,000,000)	In H_2O_2 +active catalase
10	36	—	22	22	0
40	37	—	18	31	0
50	45	—	11	27	0
40	48	46	0	—	—
40	45	48	45**	—	—
20	58	55	0	—	—
90	82	88	11	—	—

* Approximately 160,000 r.

** Sperm introduced immediately after catalase had been added to the irradiated water.

The delay in time of cleavage also was reduced by adding the catalase extract to the irradiated water or to 1 : 1,000,000 hydrogen peroxide (Table II). Apparently the reason the catalase was not 100 per cent effective in all tests was that in such instances sufficient time was not allowed before the sperm were added to the irradiated water. Maximum efficiency was obtained when the catalase had been in the irradiated water (or hydrogen peroxide solution) for approximately an hour before the sperm were added. In the one test with negative results sperm were added to the irradiated water immediately after the catalase had been introduced, and the

sperm were apparently affected before the H_2O_2 had been destroyed. The amount of catalase used was slightly in excess of that required to remove H_2O_2 (1 : 1,000,000) as determined in Warburg manometers. It is possible that in some instances the amount of H_2O_2 produced by the irradiation was in excess of 1 : 1,000,000. Strong concentrations of catalase were avoided as it was found that too much of the extract was toxic to the sperm.

TABLE III

TOXICITY OF IRRADIATED CATALASE EXTRACT

(Compared with toxicity of irradiated water)

(Toxic effects: reduction in survival time and production of cleavage delay)

Irradiation in 1,000 r	% Reduction in survival time		% Cleavage delay	
	Irradiated catalase	Irradiated water	Irradiated catalase	Irradiated water
84	24	10, 5	14	—
112	58	43, 43	20	55
140	74	58, 58	22	—
168	78	62, 62	25	70
196	80	68, 70	30	—
224	83	75, 75	35	80
140	79	—	40	—
140	74	—	30	—
140	74	—	30	—
140	87	55	43	43
140	55 (diluted to 50% with irradiated water)			
100	65	30	40	55
100	65 (diluted to 99% with active catalase)			
140	44 (diluted to 30% with untreated water)			
140	30 (diluted to 30% with active catalase)			

In an earlier investigation (Evans et al., 1942a) it was found that some egg albumin solutions afforded protection against toxicity of irradiated water whereas others did not. In checking over the data it appeared that protection was afforded only when the albumin had been prepared several days previously. Such solutions, on standing, were found to have developed large numbers of bacteria and possessed catalase activity. Fresh albumin solutions had no catalase activity and did not protect the sperm from the toxic action of irradiated water. Fresh albumin did have a protective effect when present with sperm during irradiation (Evans et al., 1942a). The albumin particles apparently competed with the sperm for the limited number of activated water molecules available and thus reduced the amount of injury to the sperm.

Toxicity of irradiated catalase extract

One type of control in the above experiments (Tables I and II) was the addition of inactive catalase to the irradiated water in which case the toxicity was not reduced. This extract had been made inactive by heating at 60–65° C. for 30 minutes.

It had been found [3] that a dose of 140,000 r would completely inactivate this concentration of catalase, so such an irradiated extract was added to heavily irradiated water to determine whether it would reduce the toxicity. The irradiated catalase failed to reduce the toxicity of the irradiated water, and had even a slightly greater effect in reducing the survival time of the sperm. These results are shown in Table III. Addition of active catalase (two experiments) did not remove the toxicity of the

TABLE IV

EFFECTS OF IRRADIATED ALBUMIN SOLUTION

Exp. No.	Material	½ Survival time in min.	Min. cleavage delay
129	(A) Sperm in control sea water.	70	0
	(B) Sperm in irradiated sea water.	20	15
Irradiation = 160,000 r	(C) Sperm in irradiated sea water + albumin later.	20	20
	(D) Sperm in *irradiated albumin solution.*	48	22
	(E) Sperm in control sea water + albumin.	1,005	0
		(% Survival at 30 minutes)	
20	(E) Control water + albumin.	96	0
Irradiation =	(C) Irradiated water + albumin.	59	40
224,000 r	(D) *Irradiated albumin.*	57	32
19	(E) Control water + albumin.		0
Irradiation =	(C) Irradiated water + albumin.		42
224,000 r	(D) *Irradiated albumin.*		42
21	(A) Control water.		0
	(B) Irradiated water.		27
Irradiation =	(F) Irradiated water which had contained		5
64,000 r	1:100 sperm during irradiation.		
	(G) Irradiated sperm removed from (F). (Direct effect of irradiation.)		85

radiation-destroyed enzyme extract. Although these results were not conclusive it was indicated that all of the toxicity of the irradiated catalase extract was probably not due to hydrogen peroxide, in other words that some other toxic materials were probably formed. A 0.1 per cent solution of egg albumin was irradiated to determine whether it would be made toxic and the results are shown in Table IV. The tentative conclusion was drawn that the albumin present in the water during irradiation had little effect on the production of hydrogen peroxide by the irradiation, but that albumin itself did not contribute to the toxicity of the solution.

Effects of irradiated water and H_2O_2 on ova

Arbacia eggs placed in irradiated water (168,000 r) or in dilute solutions of hydrogen peroxide in sea water exhibited effects similar to those of sperm so treated. These results are shown in Table V. It was found that immersion in irradiated water or in hydrogen peroxide increased the time for first cleavage and decreased the percentage fertilized. It is of interest that the effects were not as pronounced if

the number of eggs in the experimental medium was doubled. This probably indicates that the number of toxic molecules was limited and the eggs were sensitive to even small changes in the concentration of the toxic materials.

Table V

Effects of Irradiated Water and Hydrogen Peroxide on Ova

(Eggs in experimental medium, inseminated with control sperm)

Experiment 22	Control eggs in sea water	Eggs in irrad. water	Eggs in H_2O_2 (1:1,000,000)	Eggs in H_2O_2 (1:100,000)
Cleavage time: (tested after 30 min. in solution.)	42 min.	48 min.	47 min.	
% Fertilization:	100 fert.	73 fert.	100 fert.	55 fert.
Cleavage time: (tested after 90 min. in solution.)	42 min.	49 min.	48 min.	
% Fertilization:	99 fert.	69 fert.	100 fert.	

Experiment 23	*N* no. eggs in control sea water	2*N* no. eggs in control sea water	*N* no. eggs in (1:100,000) H_2O_2	2*N* no. eggs in (1:100,000) H_2O_2	*N* no. eggs in irrad. water	2*N* no. eggs in irrad. water
Cleavage time: (tested after 25 min. in solution)	47 min.	46 min.	135 min.	103 min.	46 min.	45 min.

Experiment 24	2*N* no. eggs in control sea water	*N* no. eggs in (1:1,000,000) H_2O_2	2*N* no. eggs in (1:1,000,000) H_2O_2	*N* no. eggs in irrad. water	2*N* no. eggs in irrad. water
Cleavage time: (tested after 50 min. in solution.)	46 min.	53 min.	45 min.	52 min.	45 min.
% Fertilization:	100 fert.	100 fert.	100 fert.	56 fert.	100 fert.

Discussion

These results are in agreement with the excellent work of Taylor, Thomas, and Brown (1933) and indicate that when test objects are heavily irradiated (around 100,000 r) in water, the possibility of an indirect effect through production of hydrogen peroxide must be taken into account. Conditions of the medium which have been found to affect the amount of hydrogen peroxide remaining in an irradiated solution are (1) the oxygen content, (2) the pH, (3) temperature, (4) radiation intensity (Fricke, 1934a, 1934b, 1935; Risse, 1930), (5) presence of substances which remove the H_2O_2 (Taylor, Thomas, and Brown, 1933), and (6) the amount of energy absorbed (Bonet-Maurey and Frilley, 1944).

Interference of radiation-produced hydrogen peroxide was reduced to a negligible amount in the earlier investigation (Evans et al., 1942a) by using such dilute suspensions of sperm that they were inactivated by doses (from 2,000 r to 10,000 r) too low to produce detectable amounts of hydrogen peroxide. Also, as hydrogen

peroxide in low concentrations acted only after a time, its toxicity was avoided by removing the sperm immediately from the irradiated water. Taylor, Thomas, and Brown (1933) reduced the toxicity by adding substances possessing catalase activity during the irradiation. In the present investigation, results of attempts to reduce the hydrogen peroxide content by presence of catalase extract during irradiation were puzzling in that the medium possessed toxicity, and it was not removed by active catalase. This finding does not necessarily indicate that irradiation of catalase produces toxic by-products, as many other substances were probably present. However, irradiation of water containing living sperm did not result in toxicity of the medium (Experiment 21-F, Table IV). Other data have been obtained [3] which indicate that catalase *in vivo* is not as easily destroyed by radiation as the catalase extract *in vitro*. Still another possibility is that so many activated water molecules transferred their energy to living sperm that only few remained to produce H_2O_2. Presence of 0.1 per cent albumin did not appreciably affect the production of hydrogen peroxide (Table IV), but possible competing action at higher concentrations was not studied. Catalase extract added to dilute sperm suspensions afforded some protection against the effect of the activated water molecule as studied in the earlier investigation (Evans et al., 1942a). This protection during irradiation was apparently a competing action rather than due to removal of hydrogen peroxide as the active enzyme was no more effective than heat-inactivated catalase extract. The effectiveness of a protecting substance has been indicated by a ratio of the "Median Effect Dose" for sperm in water plus substance over that of sperm in sea water alone. If active catalase is designated as A and inactive catalase as B, the ratios were as follows: Experiment 1, $A = 1.12$ and $B = 1.19$; Experiment 2, $A = 1.6$ and $B = 1.53$; Experiment 3, $A = 2.7$ and $B = 2.6$.

In the previous investigation (Evans et al., 1942a) it was concluded that cleavage delay was caused directly by the radiation, whereas fertilizing capacity (and viability) was reduced indirectly through action of an intermediary (activated water molecule). It is interesting to consider that in the present investigation it was demonstrated that, by producing hydrogen peroxide, the activated molecule could indirectly affect cell division as well as cell viability. The direct effect of the radiation on cleavage delay is considered as the loss of some factors from the nucleus which is replaced in time in irradiated eggs, but not in irradiated sperm through metabolic activity (Henshaw, 1940, I–V; Lea, 1946). Recent discussions of radiation inhibition of cell division (Hevesy, 1945; Mitchell, 1943; and Lea, 1946) suggest the possibility that the effect is due to disturbances in nucleic acid and carbohydrate metabolism by means of enzymatic inactivation. It is generally accepted that hydrogen peroxide poisons many enzymes and that the presence of catalase in cells removes the hydrogen peroxide as it is formed in metabolism. The writer has recently investigated the possible relation between radiation destruction of catalase and radiation production of cleavage delay, but the results indicated that the effects were not necessarily correlated.[3] However, one may still consider that, if both H_2O_2 and radiation retard the functioning of systems which regulate cell division rate, their mode of action may be similar (i.e., oxidation) in producing this effect. The question as to how the active agent in irradiated water (hydrogen peroxide) might have exerted its effect of reducing survival time leads one to consider factors which affect longevity of untreated sperm. This subject has been studied recently by Hayashi (1945–1946) who concluded that a factor prolonging

the duration of fertilizing capacity of Arbacia sperm was adsorbed on the cell surface, and subsequently was lost into the surrounding medium. Seminal fluid contained a protein which, by its action on the surface of the sperm, may maintain fertilizing capacity and respiratory rate. Some findings in the present investigation suggest that the specific protein complex on the surface of the sperm is present even when removed from the seminal fluid by washing and centrifuging, and that its destruction can be retarded by non-specific proteins. This is indicated by the observation that sperm, after removal of seminal fluid, could be kept alive and fertile for extended periods of time (even for days) as long as they were closely packed. In sea water the factor was lost and disappeared more rapidly the more dilute the suspension. It may be noted in the earlier report (and in Table IV, Experiment 129 of this report) that addition of a non-specific protein (egg albumin) increased the survival time of sperm in sea water. Two additional experiments will be cited. In one experiment 1 : 1,000 sperm in 0.1 per cent albumin retained their fertilizing capacity 2.6 times as long as the same concentration of sperm in sea water alone. Sperm in sea water plus albumin, in the other case, remained fertile 14 times longer than sperm in sea water without the albumin (this is the same value as obtained in Experiment 129, Table IV). Sperm in water plus catalase extract remained fertile 1.8 times as long as sperm in water alone. This prolonging of viability was not due to the enzyme activity as heated catalase extract had exactly the same effect (80 per cent increase in survival time). It may be of interest to mention in this connection the finding of Saul and Nelson (1935) and Adams and Nelson (1938) that when purified preparations of invertase or tyrosinase are highly diluted they lose activity. If serum albumin, etc., is added during the dilution, or immediately thereafter, this loss in activity is prevented and activity measurements run proportional to the dilution factor. Hayashi (1946) considers that the fall in activity of Arbacia sperm in sea water is due to destruction of a system involving the cell surface, to auto-intoxication and probable depletion of fuel. One type of auto-intoxication could be auto-oxidation (production of H_2O_2). If this is true then the irradiated water may act by removing catalase from the surface which would, in turn, allow accumulation of H_2O_2 formed during respiratory metabolism. Action of the activated molecule could be due to direct oxidation of materials at the cell surface or to destruction of protective catalase.

Summary

Heavily irradiated water (over 100,000 r) was found to have deleterious effects on sperm placed therein. These effects were reduction of survival time and delay in first cleavage when such treated sperm were used to inseminate fertile eggs. The injury became more pronounced the greater the irradiation dose and the longer the sperm remained in the irradiated water.

The chief, if not the only, agent responsible for these effects of irradiated water was hydrogen peroxide. This was shown by chemical test, by similarity of its action with that of hydrogen peroxide, and by removing the toxicity with catalase extract.

The effects of hydrogen peroxide on fertility and on subsequent cleavage time are discussed regarding possible interpretations of similar reactions of the sperm to more direct effects of roentgen radiation.

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DECOMPOSITION AND REGENERATION OF NITROGENOUS ORGANIC MATTER IN SEA WATER.* VI. THE EFFECT OF ENZYME POISONS

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When organic matter, in the form of natural marine plankton, is allowed to decompose in sea water in the dark under laboratory conditions ammonia is first found in the water, followed by nitrite, and later by nitrate, in amounts corresponding to the nitrogen which disappears from the suspended particulate matter. When, in the last stage, the water is inoculated with fresh diatoms a new flowering takes place in the light, with the disappearance of nitrate from the water. In darkness, this new organic matter again decomposes and the cycle repeats itself. Many of the features of this cycle, and the effects of such variables as time, temperature, source of water and organic matter, etc., we have reported in previous papers (1937–1942).

To renew these studies, after an enforced delay of three years, we chose to investigate the effects of certain enzyme poisons on the several stages of the decomposition and regeneration cycle. The plan was to start decomposition "cultures" consisting of natural sea water and marine phytoplankton material and to follow the ammonia and nitrite in the water, in control portions as well as in portions to which enzyme inhibitors were added at various stages in the cycle.

Since the effects to be observed were at most semi-quantitative it was possible to simplify the analytical techniques to reduce the laborious character of the ammonia analyses and to eliminate the determination of nitrate and particulate nitrogen. The distillation procedure for ammonia was identical with that previously described, but after collecting the distillate the ammonia in it was finally determined by Nesslerization rather than by titration with hypobromite. While not as accurate, this procedure was sufficient for the purpose and more rapid. Nitrite was determined by the same colorimetric method as before, with the use of the Klett-Summerson photoelectric colorimeter in the later stages of the work.

Experimental

Four decomposition "cultures" were prepared (Nos. 101, 103, 104, and 105) from water obtained in Vineyard Sound near Woods Hole. Phytoplankton was collected in a double net, so that the larger organisms were eliminated. The tow consisted principally of *Rhizosolenia*. Varying, undetermined quantities of this material were added to the water in 5-gallon carboys, No. 104 containing the largest amount and No. 103 the least. Smaller portions (about 2 liters) were removed to separate containers and the inhibiting substances added in fixed concentrations. These were allowed to decompose in the dark, at laboratory temperature, simultaneously with the main "control" portion of each culture. At intervals, determina-

* Contribution No. 371 from the Woods Hole Oceanographic Institution.

tions of ammonia and nitrite were made and at certain times portions were again removed from the main culture and inhibitors were added.

The enzyme poisons used were: KCN, in concentrations from 0.0001 to 0.01 M; NaF, 0.01 and 0.03 M; As_2O_3, 0.01 M; sodium iodoacetate, 0.01 M; ethyl, propyl and *n*-butyl carbamates, 0.2 M. These were rendered neutral by addition of acid or alkali as required and were added at various stages in the cycle: at the beginning; when the ammonia had reached a maximum; and when the ammonia had been entirely replaced by nitrite.

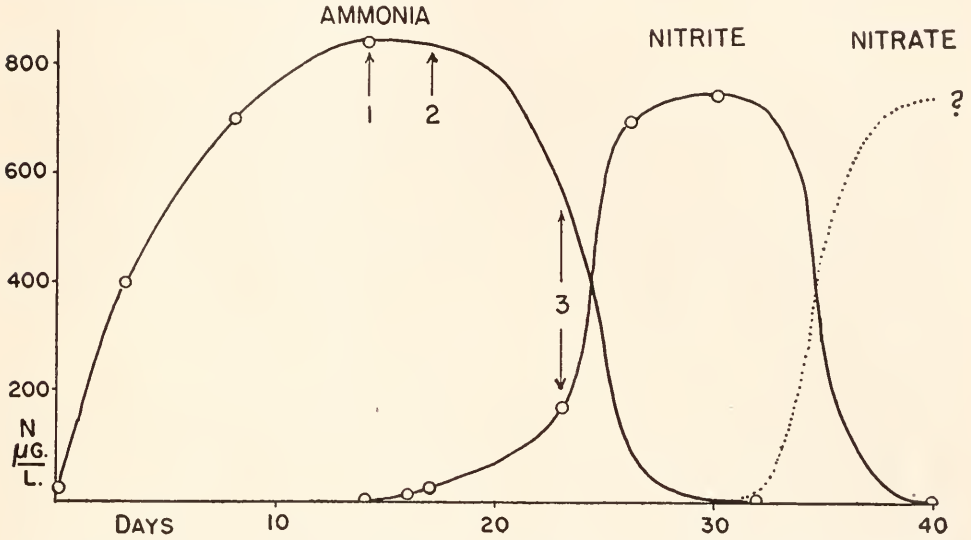

FIGURE 1. Series No. 104. Formation of ammonia, nitrite (and nitrate by inference) during decomposition in the dark. Source of organic matter, principally *Rhizosolenia* suspended in water from Vineyard Sound. Time in days, nitrogen in micrograms per liter. At points indicated by *1*, *2*, and *3* portions of this control culture were removed and to them were added various enzyme poisons, as described in the text.

Figure 1 shows the course of the cycle in Series 104. In the control, ammonia reached its maximum after about 15 days, at which time nitrite was first detected. The latter reached its maximum about 15 days thereafter, and in another 10 days—or 40 days from the start of the decomposition—it had again disappeared entirely. We know from previous experience that complete oxidation to nitrate has taken place at this time. At the beginning of the experiment five 2-liter portions were removed, to which were added respectively: *A*, KCN, 0.01 M; *B*, KCN, 0.001 M; *C*, KCN, 0.0001 M; *D*, NaF, 0.03 M; *E*, As_2O_3, 0.01 M. At the point marked *1* in Figure 1, at 14 days, portions were removed to which were added: *F*, KCN, 0.01 M; *G*, KCN, 0.001 M; *H*, As_2O_3, 0.01 M. At point *2*, at 17 days, similar sub-cultures were prepared containing: *J*, ethyl carbamate, 0.2 M; *K*, propyl carbamate, 0.2 M; *L*, sodium iodoacetate, 0.01 M. At point *3*, sub-cultures were prepared containing: *M*, KCN, 0.001 M; *N*, KCN, 0.0001 M.

Series 101 and 103 were carried out in the same manner, except for variation in the times at which the inhibitors were added. Series 105 was carried through the

first half of the cycle only, to learn the effects of the carbamates and of iodoacetate on the liberation of ammonia.

Results

The results and conclusions are based upon observation of the relative rates of appearance and disappearance of ammonia and nitrite in the presence and absence of inhibitors. The graphic details of these experiments need not be reproduced, but the following observations were made as to the specific effects of the various poisons.

KCN

In no case did significant amounts of nitrite appear in any culture to which KCN was added, even in concentrations as low as 0.0001 M. When KCN was added, even in the lowest concentration, to cultures in which nitrite had already appeared, the latter did not increase in amount, showing that the oxidation of either ammonia or nitrite was inhibited. In 0.0001 M concentration it had no effect upon the formation of ammonia, but in concentration of 0.001 or 0.01 M it greatly retarded the rate. In Series 104, for example, in which ammonia had disappeared in about 30 days in the control culture, less than half the maximal amount had appeared in 46 days in the presence of 0.01 M KCN.

Carbamates (ethyl, propyl, n-*butyl)*

When carbamates were added in 0.2 M concentration to cultures already containing ammonia no nitrite formed. If nitrite had already appeared before carbamate was added it did not further increase. Carbamate therefore appears to inhibit the oxidation of both ammonia and nitrite. It was not possible to determine the effect of carbamate upon the formation of ammonia since it interfered with the analysis, decomposing during the distillation and also rendering the Nessler reagent inactive.

Iodoacetate

Iodoacetate inhibited both the formation and further oxidation of nitrite, exactly as cyanide and carbamate. A single experiment indicated that it permitted the formation of ammonia, but somewhat more slowly than in its absence.

NaF

In a concentration of 0.03 M sodium fluoride did not interfere with ammonia formation, but in this concentration, as well as in 0.01 M, it prevented oxidation to nitrite. It should be mentioned, however, that these concentrations produce a copious precipitate of CaF_2, so that the resulting concentration of fluoride ion is considerably smaller. The solid precipitate itself may also be a disturbing factor.

As_2O_3

Nitrite did not appear when As_2O_3 was present. Its effect upon ammonia formation was inconclusive; in one case ammonia appeared but in another it did not. It proved difficult to keep the solution neutral, however, and in at least one case a strong acid condition developed which was not detected until the close of the experiment.

Discussion

Microorganisms may produce ammonia through various chemical mechanisms: hydrolytic deamination, reductive deamination and oxidative deamination (Waksman, 1932). Depending upon conditions (for example, aerobic or anaerobic surroundings) and the species of microorganisms present one or the other process will predominate. It is consequently not surprising that variations in the response to inhibitors should occur, since the various mechanisms will involve different enzyme systems. The different response to As_2O_3 observed in two series of the present study can be easily understood on the assumption of the presence of different microbial floras. The non-interference of this poison with ammonia production in one of the series is not surprising; it has been found that the deamination of amino acids by kidney slices proceeds also in the presence of arsenite (Krebs, 1933). There is also a parallel to the ineffectiveness of iodoacetate; Geimann (1936) reports that it does not suppress the ammonia formation within the tissues of the medusa *Aurelia*.

The fact that cyanide retards, but does not completely suppress, ammonia formation is of interest. It is known that all cases of oxidative bacterial deaminations so far studied are inhibited by 0.005 M KCN (Stephenson, 1939). Our observations may therefore be taken to indicate that under normal conditions oxidative deaminations occur and predominate in decomposing plankton samples. They are probably catalyzed by heavy metals. In our cyanide-inhibited samples ammonia formation would then be due to one of the other mechanisms. We cannot decide at the present time whether the slower rate of ammonia formation is due to a weakly developed alternate mechanism in the same bacteria whose oxidative deamination mechanism has been inhibited. It is perhaps just as likely that such organisms have been more or less completely eliminated and that a longer time is required to build up a bacterial population capable of liberating ammonia from protein or its higher decomposition products by a mechanism that is not sensitive to cyanide.

While the production of ammonia is common to many groups of bacteria the formation of nitrite and nitrate is restricted to specialized types. Meyerhof (1916a and b; 1917) studied the metabolism of soil nitrifiers belonging to the two groups involved. He found that both are sensitive to urethanes and that the nitrate formation is also inhibited by cyanide, but he did not study the effect of cyanide upon the nitrite-forming flora. The present study confirms these observations from the corresponding floras occurring in the sea and also establishes the cyanide sensitivity of the ammonia-oxidizing flora.

The action of cyanide can very likely be ascribed to an enzymatic inhibition. This is indicated by the observation that even very small concentrations of this poison can suddenly interrupt both the nitrite and nitrate formation; i.e., inhibition takes place even when the floras have already been built up. One is not dealing, therefore, merely with suppression of the development of the necessary number of microorganisms.

It seems likely that the formation of both nitrite and nitrate is catalyzed by heavy metals. It is possible that the cytochrome system is involved. This seems to follow from the fluoride inhibition. It has been shown in a variety of biological materials that fluoride attacks the cytochrome system; the actual point of attack seems to be cytochrome *c*, rather than the cytochrome oxidase itself (Borei, 1945). It would

be of interest to study more fully the possible role of the cytochromes during nitrite and nitrate formation.

Iodoacetate and arsenite, in the concentrations employed, are primarily dehydrogenase inhibitors. The inhibition of nitrite and nitrate formation by these poisons seems to indicate that enzymes of this type are also involved. It is, of course, a well established fact that both oxidases and dehydrogenases are frequently linked in one chain and that both are necessary to complete an oxidative process.

The inhibition observed in the presence of carbamates would under ordinary circumstances strengthen the assumption of the participation of dehydrogenases, since these narcotics are usually considered as dehydrogenase inhibitors. However, in the present case one must be cautious in interpreting the results. It is a well established fact that many organic compounds are definitely toxic to nitrifiers, especially in high concentrations (Meyerhof, 1916b; Fred and Davenport, 1921). For the present, therefore, we cannot rule out the possibility of a non-specific action of the carbamates.

A practical outcome of the present study is the suggestion that cyanide, in exceedingly low concentrations, may be an effective preservative for sea water before analysis for nitrite and nitrate. Various points, however, such as the minimum concentration of cyanide, the time limits of protection, etc., must be studied before it can be recommended for routine use.

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THE BEHAVIOR AND METAMORPHOSIS OF THE LARVA OF BUGULA NERITINA (LINNAEUS): EXPERIMENTAL MODIFICATION OF THE LENGTH OF THE FREE-SWIMMING PERIOD AND THE RESPONSES OF THE LARVAE TO LIGHT AND GRAVITY

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McDougall (1943) noted that the larvae of *Bugula neritina*, when liberated in finger-bowls containing sea water at room temperature (25–30° C.), swam without exception towards the source of illumination and within an hour the majority became attached to the surface film or to the sides of the container. The larvae remained positively phototropic throughout their pelagic stage. While experimenting with light boxes containing chambers arranged at the same horizontal level but receiving different amounts of illumination, however, he found 1,533 settings in the two chambers receiving the least amount of illumination, but only half as many in the two most brightly lighted ones. Furthermore, on tiles submerged in Beaufort Channel the most intense vertical distribution of attached larvae was very near the bottom at a depth of 79–104 inches. Their numbers gradually decreased up to a point 6 inches below mean low watermark; presumably the inability of *Bugula* colonies to tolerate exposure to air for more than a few minutes accounts for their absence above this level. These observations indicate that larvae in their natural environment become photonegative and geopositive some time before attachment. McDougall (1943) called attention to the marked contrast between the behavior of the larvae under laboratory conditions and their apparent behavior in nature.

The studies reported in this paper were undertaken to determine what environmental factors may be responsible for the observed distribution of adult colonies at Beaufort, North Carolina. Incidentally they led to an investigation of the mechanisms involved in causing metamorphosis.

The general literature on the development and metamorphosis of the Bryozoa is fairly extensive. A monograph by Barrois (1877), reviewing the behavior, development and metamorphosis of representative types of both endoprocts and ectoprocts, is the most comprehensive single treatise in the literature. Other important contributions have been made by: Schneider (1869), Claparède (1870), Nitsche (1870), Metschnikoff (1871), Allmann (1872), Salensky (1874), Schmidt (1876), Hatschek (1877), Joliet (1877), Repiachoff (1877), Barrois (1879; 1882; 1886), Hinks (1880), Harmer (1885; 1887; 1903; 1931), Ostroumoff (1885; 1886), Vigelius (1886), Pergens (1889), Prouho (1890; 1892), Seeliger (1890; 1906), Calvét (1900), Kupelwieser (1905), Marcus (1921; 1926a, b, c,), Waters (1925), O'Donoghue (1926), B. H. Grave (1930), and Rogick (1939).

Although a considerable amount of experimental work has been done during the past fifteen years on the behavior and metamorphosis of larval ascidians and a few

other sessile organisms, only a few observations have been recorded for the Bryozoa. The swimming movements of the larvae and their reactions to light have been described briefly in the earlier literature; but attempts to modify their behavior under controlled conditions have been confined largely to those of Marcus (1926a, c) and B. H. Grave (1930). The latter dealt exclusively with experimental modifications of the reactions of the larvae to light. The former, by employing vertical temperature gradients, demonstrated the existence of a fright-reaction and a lethal temperature zone at 38° C. for the larvae of the fresh-water species, *Plumatella fungosa*. He also briefly recorded the failure of these larvae to respond to an increasing oxygen-gradient and their lack of conformity to the generally accepted rules for determining the effects of viscosity and temperature on the speed of swimming movements.

McDougall (1943) discussed the breeding period, growth-rates and seasonal distribution of colonies of *Bugula neritina* in the Beaufort region and, after briefly referring to the behavior of the larvae, emphasized the need for further research. Since the embryology of this species has not yet been reported, a brief description of the larva, an account of the events that take place during metamorphosis and the duration of its various phases are presented. A review of the literature shows that the earlier embryologists only vaguely described the length of the post-fixational sequences of other species of Bryozoa. Among more recent writers, B. H. Grave (1930) has given an excellent description of the larvae of *B. flabellata* and *B. turrita* from the Woods Hole region and has recorded the growth rates during the later embryological stages, but his account of the early phases of metamorphosis is somewhat brief.

Distribution of Bugula Neritina

This semi-tropical species is abundant in the Dry Tortugas, Florida (Osburn, 1914), in Japan (Miyazaki, 1938), in Hawaii (Edmondson, 1944) and on the coast of California (Robertson, 1905). Canu and Bassler (1929) found it in the Philippines; Verrill and Smith (1874) have recorded it for the Bermuda Islands, Calvét (1900) for the southern coast of France and Osburn (1927) for Curaçao. Although it apparently does not range farther north on the Atlantic Coast than Beaufort, North Carolina, it is replaced along the northern half of the East Coast by a closely related species, *Bugula turrita,* which can be distinguished from it by the presence of avicularia.

The adult organisms are purplish-brown, branching colonies, which may reach a length of 10-12 cm. McDougall (1943) found that the vertical distribution of these colonies was most dense at a distance ranging from 6 inches to 3 feet below mean low water. Colonies near the surface were longer than those nearer the bottom and growth was more luxuriant. The distribution of adult colonies, therefore, differed considerably from that of the larvae. The writer found the bottom of a large raft to be the most suitable place for collecting adult specimens, which grew luxuriantly in close association with the ascidian, *Perophora viridis. Pennaria,* which is frequently associated with *B. neritina,* grew abundantly along the sides of the raft, but was almost entirely absent from the bottom. Light seems to be a controlling factor in causing the former organisms to attach and grow in deeply shaded regions and the latter in places exposed to bright sunlight. Along the western coast of North America, *B. neritina* becomes the dominant pile-dweller

during November, when it replaces the formerly predominant algal community, with which it is closely associated (Scheer, 1945).

Since *B. neritina* grew abundantly near the laboratory on rafts and pilings within arm's length of the surface, it was surprising to find a much altered situation less than a mile up the mouth of the Newport River. Here Bryozoa could not be found at all on pilings within three feet of the surface. Scrapings from regions lower down failed to bring up any specimens. A few sparsely growing colonies were found near the bottom of submerged objects that could be raised to the surface from a depth of 6 or 8 feet. Since samples of the water showed a surprising decline in salinity within a range of half a mile, it was considered probable that the salt content might be a controlling factor in bringing about the observed horizontal distribution in this region.

Materials and Methods

Since the histological details of the development of several species of Bryozoa are well-known, the writer used only living material for the embryological observations and the experimental work. Through the facilities offered by the U. S. Bureau of Fisheries, Beaufort, North Carolina, the larvae for these studies were obtained from sexually mature colonies placed in finger-bowls and kept in running water. These colonies usually yielded a considerable number of larvae each day for a period of ten days to two weeks. In some instances, however, larvae were produced in enormous numbers for five or six days; but at the end of that time they were no longer shed, and the colonies had to be replaced with fresh ones. During the summers of both 1944 and 1945 it was observed that there were periods lasting about a week when larvae were almost unobtainable in large numbers, even when fresh colonies had been gathered the night before. These periods occurred at approximately the same time each year, towards the end of the first wek in August. Since there seemed to be no unusual variations in temperature or sunshine, the cause of this phenomenon was not determined.

Although it would be preferable, indeed, to use only larvae from a single colony for each set of experiments, nevertheless the small number that would be obtainable by such a procedure practically precludes the attainment of such a desideratum. Some of the variations in the behavior of the larvae under similar experimental conditions may be attributable, perhaps, to differences in genetic strains. It seems more likely, however, that the length of time that the larvae have remained in the ovicells before being shed is a more important factor in introducing seemingly fortuitous variations in the length of the free-swimming period. The larvae, as a rule, show a remarkable uniformity in their behavior under similar environmental conditions; genetic variations seem to play only a minor role in determining the length of the free-swimming period.

For observations on the responses of the larvae to light and gravity at reduced temperatures, stender dishes 5 cm. in diameter and 3 cm. deep were used exclusively during the first summer. During the second summer, homeopathic vials 8 cm. long and 1.5 cm. in diameter were used for these observations and for those on the effects of salinity. For most of the light experiments, vials 16 cm. long and 1.8 cm. in diameter were employed. These were scrubbed carefully with soap and water and then rinsed thoroughly with sea water. A light-box, $8 \times 8 \times 30$ cm., was

constructed so that light could enter only one end of a vial placed within it. Well-slides were used for microscopic work.

Shortly before the experiment was to begin, finger-bowls containing adult colonies were placed near a window that admitted diffuse daylight to the laboratory. When larvae were liberated, they swam immediately towards the side of the dish nearer the window and collected there in swarms just beneath the surface film. They could then be pipetted to the various experimental vials, which were numbered so that a record of the time of placement could be kept. Since the liberation of larvae is photoperiodic, generally beginning 30–40 minutes after the parent colonies are exposed to light, active specimens could be obtained throughout the day, if the containers were placed in a darkroom on the preceding evening and removed to a window a short time before the larvae were needed. Although active swimmers could be obtained as late as 3:30 P.M. (E.S.T.) by this method, the number of larvae shed in the afternoon was generally small in comparison with the number liberated between 7 A.M. and noon. Since the darkroom was not equipped with a system of running sea water, the food supply of the adult colonies may have been inadequate under these conditions for the proper development or liberation of larvae. The finger-bowls were generally kept in the aquarium, which was situated in a fairly well-shaded spot; only on days when the room was flooded with light from an early hour were the larvae shed in large numbers before the experiment began. Observations were usually made between 7A.M. and noon, although in some cases they had to be carried out until 6 P.M. and in one case until 10 P.M. Larvae were most abundant during the first two hours after exposure of the parental colonies to light.

Observational Section

Structure of the larva

The larva of *B. neritina* resembles other larvae of the same genus in its structural features and its general contour. It may be described as pyriform rather than peach-shaped, however, with average dimensions 0.20–0.22 × 0.27–0.30 mm., although smaller specimens were observed at times. At the narrower end of the body the convex apical organ, the "calotte" of Barrois (1879), is clearly set off from the rest of the larva by a crown of rigid cilia and by a circular groove or collarette, the pallial furrow (Fig. 1). Its coloration is somewhat lighter than the rest of the larva, which looks green by reflected light, but brown by transmitted light because of the numerous pigment granules imbedded in the body. The middle of the apical organ has a darkly pigmented spot whose center shows a tiny clear area that sometimes disappears when the surrounding tissue suddenly contracts (Fig. 4). This spot marks an opening into a shallow cavity in the interior of the larva. The broader end, which corresponds to the oral depression of larvae that have a rudimentary or functional alimentary canal, contains in its center an invagination forming the relatively voluminous internal sac or sucker, the "saugnapf" of Pergens (1889), whose opening to the outside is marked by a ring of black pigment (Fig. 3). The internal sac becomes so distended by a secretion of granular material from its walls that the whole region between the equator and the oral depression bulges like the broader end of a pear. The median furrow, which B. H. Grave (1930) called a lateral groove, is clearly defined by its lighter coloration and by the absence of pigment (Fig. 2). In the median furrow near the equator is a tuft of four, long,

blunt vibratile flagella that beat in unison. This is the "plumet ciliaire" of Barrois (1877). The median furrow and its associated glandular structures constitute the piriform organ. In some specimens there are two, small, darkly pigmented eye-spots near the tuft of flagella and symmetrically placed with reference to the median furrow. They can be seen from the opposite side through the somewhat transparent larva (Fig. 1). On the side of the larva opposite the median furrow are two, prominent, black, diamond-shaped eye-spots lying almost on the equator and about 90° apart. These structures are a constant feature of the larvae and serve as excellent landmarks for their proper orientation during swimming movements. A tiny, white area that appears frequently in the center of each eye-spot, when light falls at the proper angle, is somewhat difficult to interpret; probably it is caused by a concentration of light brought about by a crystalline lens that covers the eye-spots, as Nitsche (1870) has described for another species of *Bugula*. The locomotor cilia, which cover the body except in the region of the apical organ, are more active near the apical organ than on the half of the body containing the oral depression; if stationary larvae are observed, water currents can be seen flowing from the region near the apical organ to the equator and then outwards and back again to complete a circle. Since locomotor cilia of most bryozoan larvae are found only on the enlarged coronal cells that form an elevated ring around the body of the organism, Barrois (1877) has interpreted the presence of cilia on the whole body of *Bugula* larvae as an indication that the corona has spread out enormously in these forms, so as to occupy the whole region between the apical organ and the oral depression. This view is supported by the fact that the corona of *Flustrella hispida* is a single band around the oral pole, whereas it is separated into two distinct bands in the larva of *Cyphonautes compressus* and in other forms having a similar type of larva. It is quite possible, however, to regard the condition in *Bugula* as primitive; condensation of the cilia into bands may not have occurred until a later period of phylogenetic development.

Changes in body contour, accompanied by elongation of the larvae, can be observed frequently. In some cases larvae look lobular rather than pear-shaped, as though they were divided into four lobes by two deep constrictions running at right angles to each other (Fig. 5). This appearance results from a contraction in the region of the equator accompanied by a depression of the apical organ.

Orientation and swimming movements of the larva

The larvae usually swim with their long axes tilted at an angle of 45° with the vertical, if they are advancing in a definite direction and are not in contact with the bottom of the container or the surface film. Both the apical organ and the tuft of vibratile flagella are in advance and the median furrow is directed downward. In this respect the larvae differ from those of *B. flabellata,* which swim with their long axes directed horizontally, and from the larvae of the Escarina, in which the long axes are vertical (Barrois, 1877; 1886). Spiral movements, however, can be observed frequently. While spiralling, the organism usually has its median furrow on the outside of the spiral. This is probably why B. H. Grave (1930) called it a lateral groove. (He clearly described the function of the flagella and cilia in these movements.) If the larvae are in contact with parts of the container or the surface film, they progress by creeping movements with the median furrow and the vibratile flagella in contact with the surface. Sometimes, while on the bottom, they spin on

their long axes and show in quick succession first the median furrow and then the pigmented eye-spots.

Observations of these diverse methods of locomotion have brought about a certain amount of confusion in the literature on closely related species. Calvét (1900), for instance, in referring to the larva of *B. sabbatiere* described the half containing the eye-spots as posterior and the other half bearing the median furrow as anterior. B. H. Grave (1930), on the other hand, referred to the apical organ of *B. flabellata* as the anterior end. His orientation is in agreement with that of Barrois (1877). As Grave (1930) noted, it is necessary to distinguish the anterior half of the larva, as determined by its forward motion, from the morphological anterior end; for the mouth, when one is present in bryozoan larvae, is opposite the apical organ. Barrois (1877) cited similar cases showing the different interpretations of various observers regarding the orientation of the cyphonautes larva of *Membranipora pilosa* (*Electra pilosa*) (p. 232). It seems worth noting that the sensory structures, the apical and piriform organs, are in advance as the larva moves forward and first come in contact with the environment. According to Prouho (1890) the apical organ of *Flustrella hispida* is connected to the piriform organ and also to the cells of the corona by nervous tissue. There can no longer be any doubt about the sensory nature of either the apical or piriform organs.

During the early part of the larval period, relatively long distances are covered by a spiralling motion. Finally there is a slowing down of movement, accompanied by a change in the method of progression. Spiralling ceases, and the larvae now swim in circular planes parallel to the substrate; the radii of the concentric circles formed by the larvae decrease in length as the onset of metamorphosis approaches. Eventually they slow down enough to be kept within the low-power field of the microscope. While swimming in circles, larvae often behaved as if a very fine, sticky thread trailed beneath the piriform organ, which was downward at the time. Although the thread itself could not be seen, several larvae were observed to pull debris, with considerable difficulty, at a distance of several millimeters; they could be pushed and pulled with a probe at a short distance from their bodies.

Before fixation, larvae alight on a suitable substrate and begin to rotate counterclockwise on an axis running from a point midway between the eye-spots to the opposite side, where the piriform organ touches the substrate. This continues for 5

PLATE I

FIGURE 1. Profile view of the larva showing pigmented eye-spots. 1. Convex apical organ, 2. Crown of rigid cilia, 3. Collarette or pallial furrow, 4. Eye-spots on the opposite side, seen through the somewhat transparent larva, 5. Ciliated corona, 6. Eye-spot, 7. Pigmented band.

FIGURE 2. Profile view showing the median furrow and associated structures (the piriform organ). 1. Part of the glandular system, 2. Eye-spot, 3. Tuft of vibratile flagella, 4. Median furrow, 5. Oral depression.

FIGURE 3. A three-quarters view showing the position of the long axis during spiral movements. 1. Opening to the internal sac, 2. Oral depression.

FIGURE 4. View showing the apical organ. 1. Opening in the apical organ, 2. Crown of rigid cilia, 3. Pallial furrow.

FIGURE 5. View showing the larva compressed in the region of the equator with the apical organ retracted. 1. Retracted apical organ.

FIGURE 6. Profile view, 15 seconds after fixation, showing characteristic hour-glass appearance. 1. Pallial furrow, 2. Vibratile flagella, 3. Ejected holdfast.

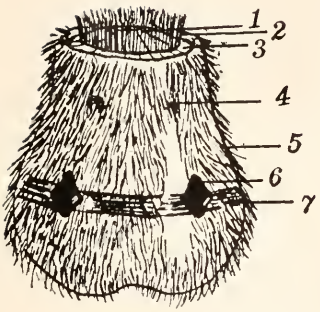

Fig. 1

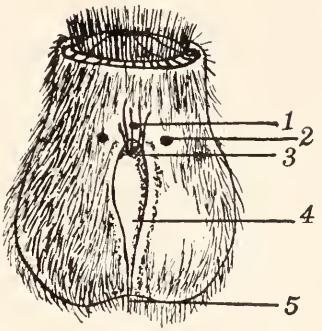

Fig. 2

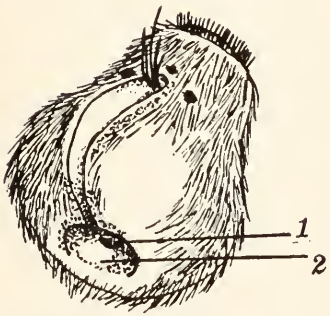

Fig. 3

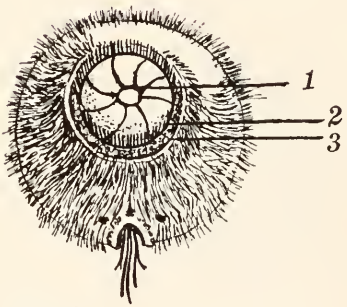

Fig. 4

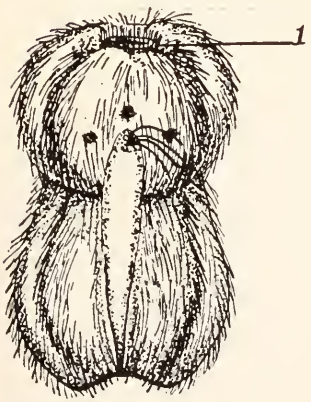

Fig. 5

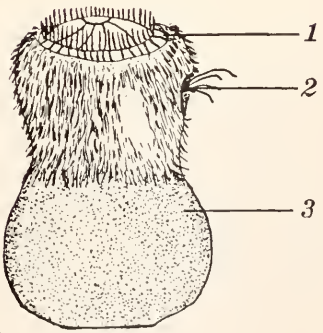

Fig. 6

PLATE I

or 10 minutes. Rotation gradually decreases in speed and finally stops just before attachment. The flagella of the vibratile plume enable the larvae to choose places suitable for fixation. Light and shadow undoubtedly play an important role in the selection of a favorable spot.

Metamorphosis

Just before fixation the larva actually appears to be anchored to the point of attachment by an invisible thread-like substance. If it is going to attach to the bottom of a well-slide, it alights with the eye-spots uppermost and the median furrow along the substrate. While it revolves slowly, always in a counterclockwise direction, it changes its shape, so that it looks like an oblate spheroid, bulging at the equator and flattened in the region of the apical organ and oral depression. Perhaps these changes in body contour indicate the establishment of a new polarity just before fixation, since Child (1925) found that a reversal of polarity occurs in hydrozoan larvae immediately before attachment.

Before setting, the vibratile flagella are very active, feeling the surface of attachment, while the larva revolves slowly for 3 or 4 minutes. At the moment of fixation the median furrow grasps the substrate by means of its ridges, which are both muscular and glandular. Within a second or two the larva elongates, so as to double the length of the long axis of its body. Then very suddenly the internal sac, containing a white, translucent, slightly granular, jelly-like material, is everted and forms a light colored, rounded mass beneath the organism, which is almost as large as the larva itself.

Simultaneously with the eversion of the internal sac, the median furrow releases its grip on the substrate and the larva rotates, so as to change the original long axis of the organism from a horizontal to a nearly vertical position. The apical organ, therefore, is brought upwards, but in such a manner that it faces away from the source of illumination at an angle of 15 or 20° from the vertical. As the larva rotates, it orients itself by squirming movements, until the eye-spots are located on the lighted side. The writer did not observe that the apical organ in this species points towards the source of illumination at the time when metamorphosis commences, as Grave (1930) has described for *B. flabellata*. After fixation the aboral

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PLATE II

FIGURE 7. "Umbrella stage" (semi-diagrammatic optical section). 1. Epithelium from the pallial furrow, 2. Holdfast.

FIGURE 8. Diagram showing epithelium from the pallial furrow forming a firm union with the holdfast. 1. Epithelium from the pallial furrow, 2. Reflected original larval covering.

FIGURE 9. The fixed larva viewed from above, 13 minutes after fixation. 1. Ringed cavity surrounded by degenerating ciliated larval covering (the corona), 2. Ciliated covering in the process of degeneration, 3. Transparent anhiste zone (the holdfast), 4. Eye-spots seen through the new larval covering (epithelium from the pallial furrow).

FIGURE 10. The fixed larva, viewed from above, 18 minutes after fixation. The horse-shoe shaped mass has increased in diameter.

FIGURE 11. The cystide, nine hours after fixation, showing a cut in the region where the piriform organ was situated. The rudiment of the polypide can be seen in the center.

FIGURE 12. The cystide somewhat older than that shown in Figure 11 (about 12 hours after fixation). The rudiment of the polypide is already well-formed. 1. Granular material being extruded into the surrounding water, 2. Break in the outer cystide wall, 3. Rudiment of the polypide.

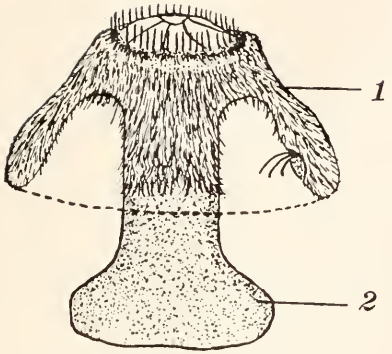


Fig. 7

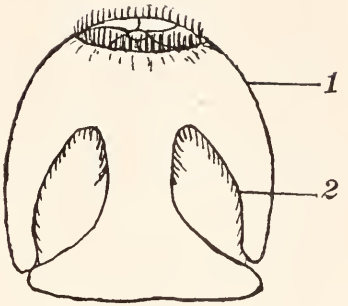


Fig. 8
(diagrammatic)

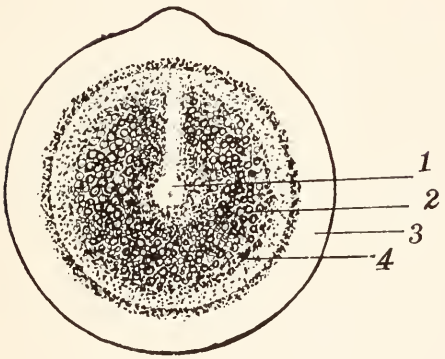


Fig. 9

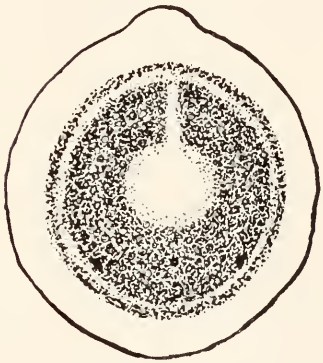


Fig. 10

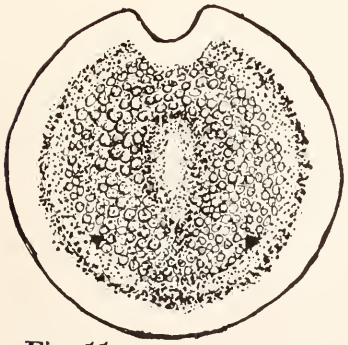


Fig. 11

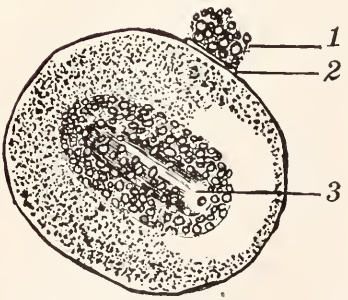


Fig. 12

PLATE II

end of the attached larva and later the apical part of the first zoöid are oriented so as to point away from the direction of the incoming light.

With the eversion of the internal sac, which formerly distended the oral hemisphere of the unmetamorphosed larva, the central portion of the body collapses somewhat and forms a narrow column supporting the apical organ on top and connecting it to the upper part of the evaginated internal sac beneath. Thus the central region of the organism becomes constricted and the larva assumes a characteristic hour-glass appearance (Fig. 6). A cavity is formed in the region formerly occupied by the internal sac. This hour-glass appearance lasts only 3 or 4 minutes, while the larva orients itself to light. If the slide is subsequently turned through an angle of 180°, no further orientation will take place; the eye-spots will remain on the side opposite the source of illumination.

Then very suddenly the apical epithelium unfolds from the pallial furrow, where it was tucked-in to form the collarette, and extends outward and downward, carrying with it the upper border of the ciliated larval covering as it migrates towards the adhesive plate (Fig. 7). This is the "umbrella stage" of Barrois (1886). The ciliated covering of the larva thus becomes reflected outward and downward, until it finally becomes completely enclosed by the apical epithelium (Fig. 8). The cells of the latter become stretched and flattened, so that their resultant transparency allows prominent landmarks like the pigmented eye-spots to be seen beneath it. The latter are carried downward during this process, until they finally rest just above the everted internal sac, where they form prominent bulges; they remain visible for 15 or 20 minutes after fixation (Fig. 9). Then with a sudden contraction the apical organ is pulled down towards the everted internal sac, which becomes flattened to form an adhesive plate. The larva now looks like a rounded cup inverted over a plate.

Just after the aboral organ is pulled down towards the adhesive plate, the fixation substance seems to grow larger in diameter, when viewed from the apical pole. This is probably caused by a flattening-out of the adhesive material and by a simultaneous constriction of the central, ciliated, papilla-like structure, which forms a ring of small diameter. At this time the opaque parts of the organism, the ciliated former outer covering and the apical organ, measure 0.14–0.15 mm. in diameter. Then the size of the adhesive plate seems to decrease again and at the same time the central ciliated mass widens, so that the clear area in the center becomes larger (Fig. 10). This appearance of a decrease in the diameter of the adhesive material can be attributed to the beginning of a union of its outer margin with the lower border of the reflected epithelium from the pallial furrow, which forms a new covering for the organism. The adhesive apparatus itself consists of a lower layer of transparent material, probably a cement-like adhesive substance formerly contained in the internal sac, and a smaller layer on top; the latter, which is somewhat pigmented, is the border of the internal sac.

The lower border of the epithelium from the pallial furrow does not form a firm union with the adhesive plate until 20 or 30 minutes after fixation. The cilia, at the margin of the former larval covering and the epithelium from the pallial furrow which covers it, may be seen beating for as long as 10 or 15 minutes after attachment. The vibratile flagella continue to move rapidly at first and then spasmodically for 5 or 10 minutes longer, until the cells to which they are attached have been carried well into the interior. Before these cells have migrated to a position nearer

the center of the larva, the flagella can be seen sticking out on top of the adhesive plate, which forms a prominent bulge in this region (Fig. 9). In many instances granular material was observed to be extruded into the surrounding water, as if the adhesive plate were ruptured by the movement of the flagella. Finally, they retreat completely within the new outer covering.

As a result of all these changes, the vibratile flagella and the associated piriform organ, as well as the whole ciliated original larval covering, have become enclosed by a new outer covering formed by epithelium from the pallial furrow. At this time the larva looks like a sac, closed on the bottom by the adhesive plate; its top and sides are formed by the apical organ and by the epithelium from the pallial furrow respectively. Its wall is lined on the inside by the ciliated former larval covering, which looks like a C-shaped mass, a ringed hole, within the larva. This C-shaped appearance can be attributed to the fact that the ciliated epithelium is darkly pigmented and opaque, except in the region of the piriform organ. What looks like an opening in the ring is undoubtedly the unpigmented piriform region, for the flagella always retreat inward from this side of the larva. After 10 minutes this ring gradually widens, the central clear area grows larger in diameter and the pigment seems to diffuse. This change probably results from the disintegration of the ciliated larval covering and of all the organs that served a useful purpose in the free-swimming organism.

Shortly after the apical epithelium emerges from the pallial furrow to form a fold which advances downward towards the adhesive plate, the apical organ loses its vesicular appearance and the tiny opening in its center begins to grow wider. During this time the apical epithelium can be seen advancing outward very slowly, as if it were being pulled and stretched by the new larval covering. This partially clarifies the observation that the upper margin of the old ciliated covering retreats inward very gradually, as the amount of material of the new covering is augmented very slowly by additions from the apical region.

Apparently the whole apical organ invaginates into the center of the C-shaped depression and undergoes histolysis, just as Prouho (1890) described for *Flustrella hispida*. He stated that the rudiment of the polypide, although it makes its appearance in this region later on, does not develop from the invaginated apical organ but rather from a meso-ectodermal disc formed independently from apical epithelium and from an internal mesodermal rudiment. The apical organ, like all the other organs that functioned in the free-swimming larva, degenerates after invagination.

Within an hour after fixation the adhesive plate, in the region where the vibratile flagella retreated, becomes deeply constricted by a notch, which can be seen for 4 or 5 hours after attachment (Fig. 11). One or two dark protrusions sometimes appear on the side opposite the notch. These are apparently the eye-spots pressing against the outer covering of the metamorphosing organism.

The larva passes into a stage known as the cystide about an hour after fixation. During this time the ectodermal walls secrete a thick covering, the ectocyst, which makes observation difficult. The further development is essentially similar to that described by Prouho (1890) for *Flustrella hispida*.

Eight or nine hours after fixation the rudiments of the polypide can be distinguished near the top of the elongating mass opposite the basal end and in the center of the now poorly-delimited C-shaped ring (Fig. 11). Twenty-four hours after fixation the zoöecium and the polypide within it are clearly recognizable. The

zoöecium itself is club-shaped, broader at the apical end where the lophophore will appear, and gradually narrowing towards the basal end, but flaring-out again at the point of attachment. At the end of 24 hours the young zoöids measure 0.6–1.0 mm. By 48 hours they have reached a length of 0.74–1.2 mm. and at 49 hours, 0.8–1.3 mm. The lower four-fifths of the body of the 24-hour zoöid is composed of transparent material derived from the adhesive plate; within it the tube-like polypide can be seen extending to the base of the holdfast. The apical end is made up of pigmented material, which forms a somewhat elongated, spherical mass that looks considerably like the unmetamorphosed larva. The transparent zoöecial wall encloses this spherical mass. At the extreme tip of the apical end two black spots mark the lophophores of the first and second individuals of the colony. The tentacles of the first are usually everted at the end of 28 hours; by this time a bud for the second individual is already well formed. Its tentacles are not everted, however, until approximately 48 hours have elapsed.

Since the lophophore and mouth of the first individual of the colony are formed in the region formerly occupied by the apical organ of the larva, there is a reversal of polarity through 180°. The oral end of the larva becomes the aboral end of the ancestrula of the colony.

The eversion of the internal sac and the events that take place during the "umbrella stage" occur so rapidly that the writer had to observe 40 or 50 larvae in order to get a clear grasp of the details. The "umbrella stage" can be seen best, when larvae attach to the lateral walls of a well-slide. On the other hand, the fimbriated nature of the holdfast can be seen only when larvae attach to the surface film. In this case the piriform organ is upward and the eye-spots are downward at the moment of fixation. Then the internal sac is suddenly everted upwards above the larva like a relatively huge balloon. The larva then turns, so as to bring the apical organ downward beneath the surface and the oral depression upward.

Larvae that attach to the surface film show no irregularities of development. The tentacles of the first individual and sometimes the second are everted in a normal manner, and they snap vigorously during active feeding. They are quickly withdrawn into the tentacular sheath, if the water is disturbed by jarring the table. When they are touched with a probe, their holdfasts cling tenaciously to it. Although the longevity of these larvae was not determined, on several occasions they were still alive 3 or 4 days after fixation. By this time the young zoöids had turned over, so that the tentacles protruded upwards. Strangely enough, they formed a continuous chain by means of thread-like projections that united each of them to the adjacent zoöids.

Although Visscher (1927) found that more bryozoan larvae affixed themselves to red test panels than to green, black or yellow ones and that none attached to white panels, the writer found no evidence that the larvae of *B. neritina* showed any preferences for pieces of red brick placed within the finger-bowls. They did prefer, however, the rougher surfaces of submerged test panels to the smoother parts, just as Visscher found. It appears likely that these larvae prefer to attach to organic material. In nature they frequently affix themselves to algae and to established bryozoan colonies. Although the presence of bacterial slime favors the attachment of most macroscopic organisms (ZoBell and Allen, 1935) and seems to be a prerequisite for the fixation of barnacles (Hillen, 1923), it does not have

any influence on bryozoan larvae (Scheer, 1945; Miller, 1946). Scheer (1945) found that bryozoan larvae would not attach to glass plates unless they had been submerged for at least two weeks; but they were influenced only by the abundant diatom population that had accumulated. The writer observed, during a three-week period in which larvae were shed daily in a finger-bowl, that 20 or 30 had attached to the body of a young *Styela* that was 2 or 3 cm. below the surface of the water, but none attached to the side-walls at this level.

Experimental Section

The effects of temperature on the responses of the larvae to light and gravity

Grave (1930) found that the larvae of *Bugula flabellata* are photopositive during the greater part of their free-swimming period but become negatively phototactic shortly before fixation.[1] Mast (1911), too, found that other organisms show a similar reversal of phototropism from positive to negative as they approach the end of the pelagic stage. The larvae of *B. neritina*, however, under ordinary laboratory conditions of temperature and salinity were never observed to become negatively phototactic at any time; swarms of metamorphosed larvae could always be found on the lighted side of the container within an hour after they had been shed. Furthermore, they maintained a definitely negative geotaxis throughout the entire larval period and attached in large numbers just beneath the surface film. In some instances they attached to the sides of the container, but fixation always took place at or near the surface. Here, then, was a paradox! What factors in nature, not operative under the unusual conditions of the laboratory, could be responsible for causing larvae to attach near the bottom of the channel and in regions not exposed to the direct rays of the sun? At first, temperature seemed to be the most likely factor, for setting of the larvae occurs most abundantly during April and May (McDougall, 1943), when the sea water is relatively cooler than it is during the summer months. Again, it seemed likely that the more normal behavior of the Woods Hole species in the laboratory might be due not so much to specific differences as to lowered temperature, for the average temperature prevailing there during May, June, July, and August is 6° below that at Beaufort.

The hypothesis that temperature might be a controlling factor in bringing about the observed distribution was first tested during the summer of 1944. Although a reduction of temperature to coincide with that of the channel during April and May did not change the geotropism of the larvae, further cooling had a marked effect. The results of two experiments, selected from many similar ones with both stender dishes and vials, are summarized in Table I, which shows the responses of the larvae to both a descending and an ascending gradient. Counts were made at approximately 2.5–3.0-minute intervals during a 30-minute period, just long enough to reverse the geotropism of all the larvae. The vials were immersed in ice-water, and temperatures were taken at the bottom, where the water was 4 or 5° cooler than on top. Although no definite temperature could be determined as the critical one at which all the larvae were either geopositive or geonegative, nevertheless the data in this table and in others not recorded show that the greatest shift

[1] The terms, phototropism and phototaxis, will be used synonymously in this paper, without regard for their original meaning as described by Mast (1911, p. 253). These organisms may be either phototactic or photopathic, according to the older terminology.

from one response to the other occurred between 20 and 23.5° C. It can be noted that larvae exhibit a kind of inertia to a change of position, when either ascending or descending temperature gradients are employed. A change in geotropism is a function of time as well as of temperature. Thus, a drop of 13° had effected a change in only 37 per cent of the larvae; the remaining 63 per cent were affected by a change of only 3°. During the last 15 minutes of the experiment when the temperature remained constant (at 7°), 35 per cent of the larvae descended to the bottom. Similarly 65 per cent had not responded to a rise of temperature from

TABLE I

Temperature	Number of geopositive larvae	Percentage geopositive
23.0	0	0
14.1	16	34
10.0	17	37
9.5	19	41
9.0	22	48
8.5	24	52
7.5	25	54
7.0	30	65
7.0	33	71
7.0	34	74
7.0	36	79
7.0	39	85
7.0	46	100

Temperature	Number of geopositive larvae	Percentage geopositive
7.0	46	100
10.0	43	93
12.0	40	87
14.0	40	87
16.0	34	73
18.0	30	65
20.0	21	45
20.5	15	33
21.5	12	26
22.0	3 (active)	6.5

7 to 18°. During a subsequent 15-minute period, however, all but 6.5 per cent had reversed their positions in the vial, even though the temperature rose only 4°. Nine larvae, having attached at the bottom, could not respond.

Not only does a reduction of temperature change the geotropism of the larvae, but it also prolongs the free-swimming period. When normal sea water was cooled to 7 or 8° C., more than 80 per cent of the larvae were found to be active at the end of an hour, in contrast to only 6.7 per cent in the controls. In general, it may be stated that the average free-swimming period can be prolonged 2–3 hours by cooling the medium. Although the extreme limit to which the natatory period could be extended by treatment with cold water was not determined, nevertheless, in several instances 20–30 per cent were found to be active at the end of 5.5 hours. If we take into consideration the fact that the majority of larvae metamorphose at room temperature within 30–60 minutes after release from the ovicells, the effects of tem-

perature on the length of the swimming period are very marked. Marcus (1926c) made similar observations on the larvae of fresh-water bryozoans.

The motility of larvae can also be modified by temperature. When quiescent larvae, which had not yet metamorphosed, were taken from containers at room temperature (28° C.) and placed in water at 7–18°, they immediately became active. Although it is difficult to judge the degrees of motility merely by observation, apparently the greatest amount of activity was exhibited when the temperature was reduced to 16°. At 12° it was still considerable, but less than at 16°. At 7° motility was still more reduced and activity was largely confined to creeping movements.

Some organisms reverse their reactions to light when the temperature is reduced. Thus, *Euglena* (Mast, 1911), *Chromulina* (Massart, 1888), *Acartia* (Esterly, 1919) and haematococcus swarm spores (Strasburger, 1878) are photopositive at room temperature (18–20° C.) but become negative when the temperature is reduced (to 4–8° C.) Others, such as *Polygordius* (Loeb, 1905), change from positive to

TABLE II

Temperature	Percentage nearer the source of illumination	Percentage intermediate	Percentage farther from the source of illumination
22	35.0	31.2	33.8
20	73.0	4.0	23.0
19	19.4	16.0	65.0
19	31.0	14.0	55.0
19	81.0	0	19.0
17	76.2	14.3	9.5
13	80.0	20.0	0
9	80.0	0	20.0
7.5	0	51.7	48.3

negative with a reduction in temperature. *Daphnia,* on the other hand, shows no reversal of phototropism when the temperature is changed (Yerkes, 1900). In order to determine the effects of various temperatures on the phototropic reactions of the larvae of *B. neritina,* the following observations were made (Table II). Contrary to expectations based on casual observations, these experiments led to the conclusion that in cold water the larvae merely lose their otherwise intense positive reaction to diffuse daylight and become more or less indifferent, swimming back and forth towards and away from a source of illumination. Although B. H. Grave (1930) had observed that this activity precedes a definitely negative reaction in *B. flabellata,* the writer found that setting of the larvae of *B. neritina* occurred more or less at random in water at reduced temperatures.

Since larvae metamorphosed, apparently without any exceptions, on the lighted side of finger-bowls containing water at room temperature, for a time it was considered that they were photopositive at the time of attachment. When small vials were used, however, fixation occurred in 70 per cent of the cases either at random or at the center of the vials in circular masses just under the surface film. Approximately 50 per cent were on the half of the disc farther from the source of illumination. These settings seem to indicate that even at room temperature larvae become indifferent to light shortly before fixation. Since phototropism is correlated

with the amount of activity of the larvae, active swimmers are almost universally photopositive; as activity decreases, they lose their intense positive reaction to light and become more or less indifferent. Because a reduction of activity occurs only a very short time before metamorphosis, larvae under laboratory conditions usually cannot move very far from the lighted side of the container before fixation takes place. Even when larvae are made to react negatively to light by experimental prolongation of the free-swimming period, as described later, this reaction is never so intense as the positive one. Perhaps this observation explains why McDougall (1943) found the highest incidence of settings not in the most dimly illuminated part of the light box, but in the adjacent chamber where slightly more light was admitted.

Phototropism vs. geotropism

Marcus (1926a) stated that the negative phototropism exhibited by the larvae of the phylactolaematous bryozoans shortly before fixation dominates a coexistent negative geotaxis and forces them to seek places of attachment at some distance beneath the surface of the water.

Since in nature both light and gravity act in approximately the same vertical plane, it might seem logical to identify a positive geotaxis with a negative reaction to light. The behavior of the larvae of *B. neritina* at reduced temperatures, however, demonstrates that they may become geopositive without showing a simultaneous negative reaction to light. The following experiments were set up to determine which of the two tropisms is dominant in this species.

TABLE III

No. of trials	12
Average temperature (mean)	28.1 $\sigma = \pm 1.1$ (25–29 degrees)
No. of larvae	332
Percentage geopositive	28
Percentage intermediate	14.2
Percentage geonegative	57.8
Average time of exposure in minutes	8.9 $\sigma = \pm 1.8$ (8 to 10 min.)

If the response of the larvae to light were dominant to their geotaxis under given conditions, they ought to swim against a gravity gradient towards or away from a source of illumination, depending on their phototropism at the time. Consequently, on several different days larvae were made geopositive by treatment with low temperatures. Vials 16 cm. long and 1.8 cm. in diameter were used; some of them were cooled to 5° C., some to 6°, and others to 8°. They were then placed inside the light box, so that rays from a 100-watt bulb could enter only from the top. The source of light was 22 cm. from the bottom of the vials, where the larvae were swimming. As might be expected from the previous discussion of the indifference towards light exhibited by larvae at reduced temperatures, a positive phototropism under these conditions could not be demonstrated. Only 2.1 per cent of the larvae in 4 of the vials swam to the top; in the other three, none changed their positions as the result of the illumination. Six trials were then made with the light-box arranged so that rays could enter only from below. The light source was somewhat nearer the larvae in these cases, being only 6 cm. from the bottom of the vials. In all cases only negative results were obtained. The larvae did not swim away

from the bottom in a negative response to light. These experiments indicate, then, that larvae at low temperatures are either indifferent to light or that their positive geotaxis under these conditions is strong enough to counteract any reaction to light.

Different results were obtained with larvae kept at room temperature. When pipetted to vials, they were geonegative at the beginning of the experiment, and the vials were then placed in the light-box so that they were illuminated only from below. Many of the larvae swam downward. Counting was done immediately after the vials were removed and the results of 12 trials are recorded in Table III. Larvae were always observed to swim upwards, when the vials were removed from the source of light, in all the trials except one; they were never seen swimming in the opposite direction. Of the 332 larvae tested only 57.8 per cent remained geonegative after treatment. In the control vials, however, 91.2 were negatively geotactic. If due allowance is made for the probability that some of the larvae in the experimental vials had metamorphosed during treatment and could not respond, the contrast in percentages would indicate that at room temperature a positive phototropism is dominant to a negative geotaxis in approximately half the larvae. On the other hand, these experiments show that larvae respond positively to light with much greater readiness when they can swim along a horizontal plane than when they must swim downward against what appears to be a buoyancy gradient. In one of the vials illuminated from below all the larvae became geopositive and metamorphosed on the bottom, but, since this vial was exposed to alternate periods of intense and diffuse light, it seems likely that a positive geotaxis resulted from the prolonged activity induced by alternate periods of light. Subsequent experiments demonstrated that any condition that prolongs the activity of the larvae eventually induces a positive geotaxis.

The effects of light intensity and darkness

Changing the intensity. A rheostat was used to govern the intensity of a 100-watt bulb placed 8 cm. above a vial enclosed by the light-box. A ten-minute exposure to dim light was followed by a ten-minute exposure to intense light. Although 15 trials were made, all attempts to bring about a downward migration of the larvae by suddenly either increasing or decreasing the intensity of illumination gave no results. Apparently the larvae of *B. neritina* are not affected by changes of light intensity, as so many plankton organisms are.

Intense light. Since strong illumination often causes a reaction in many animals which is opposite to their behavior in diffuse light, experiments were performed by using both artificial illumination and direct sunlight. The artificial illumination was at least three times as intense as that employed in the experiments described above or those summarized in Table III. Sixteen-cm. vials of normal sea water were placed 3 cm. below and at the side of a 100-watt bulb, and a water jacket was used to maintain a constant temperature of 29° C. At the beginning of the experiment larvae removed to these vials were very active, intensely photopositive and were swimming on top. By the end of 30 minutes, however, 41.8 per cent of the larvae in the experimental vials had descended to the bottom, whereas only 9.8 per cent became geopositive in the controls. Apparently a fairly high percentage of larvae at room temperature is negatively phototropic to intense light. The data obtained with strong sunlight, although comparable, were not clear-cut enough to

warrant publication, since temperature may have modified the results somewhat. Nevertheless, there is some evidence that strong sunlight causes larvae to move downward from the surface to regions of lower light intensity

Both strong sunlight and artificial illumination induce larvae to move along a horizontal plane away from the side where the rays enter the medium obliquely. Although strongly photopositive at first, by the end of 20 minutes all the larvae (in three vials) had moved away from the bulb to the distal side. Those exposed to sunlight remained photopositive much longer; but by 1.5 hours all had become photonegative in two of the vials and only 5 per cent remained photopositive in the third. In the latter, 45 per cent were intermediate and 50 per cent showed a negative reaction. Here again there is evidence that light is more effective in causing larvae to move along a horizontal plane than up or down, either towards or away from a source of illumination.

The negative reaction of larvae to intense light would seem to be significant. Even a casual observation of the distribution of various species of *Bugula* near the water line on piers and docks will show a preponderance of colonies on the north side of objects and in shaded places. The evident fact that in nature the majority of larvae are negatively phototropic at the time of attachment may be explained on the basis that they swim away from regions receiving the direct rays of the sun after a certain amount of exposure to intense light, a behavior that they do not show in a laboratory illuminated only by diffuse light. The opinion of Esterly (1919) that the physiological state of animals is changed, when they are removed from the ocean to the laboratory, would seem to be true only if all the conditions prevailing in their former surroundings are not exactly duplicated. Since the light-box used by McDougall (1943) was placed only 6 inches below low water mark, the amount of absorption of the sun's rays was probably insignificant; hence it should be expected that the larvae would be photonegative as described. Whether larvae far beneath the surface at the time of attachment show a negative phototaxis like those near the water-line can only be conjectured. Since absorption would considerably reduce the intensity of sunlight received by the lower regions, it might be expected that they would be either photopositive or indifferent, like those in the laboratory under conditions of diffuse daylight.

Darkness. Darkness sometimes inhibits the movements of animals and may change their reactions to both light and gravity. The work of Holmes (1905) on *Ranatra* led him to conclude, "The causes that produce the negative reaction [to light] are, as a rule, those which lead to diminished activity and excitement. Cold, exposure to darkness, and the quieting effect of contact stimuli lead to a condition of lessened excitability and, perhaps as a result of this, to a negative reaction to light" (p. 317). Mast (1911) obtained a reversal of phototropism in the larvae of *Arenicola* by subjecting them to a variety of agents, such as diluted and concentrated sea water, magnesium chloride, atropin, sodium hydrate and ammonia. He believed that depressants merely hasten the onset of that distinct physiological state, usually accompanied by a negative reaction to light, which larvae normally attain as they grow older. Esterly (1919) found surface specimens of *Acartia* to be negatively geotropic in diffuse light but positive in darkness at the same temperature, although deep sea specimens (at room temperature) showed no change in geotaxis. Marcus (1926a) found that the larvae of *Plumatella fungosa*

are negatively geotactic in nature, both in darkness and in light, throughout the entire natatory period.

In a series of experiments, larvae of *B. neritina* were placed in stender dishes at room temperature and removed to a darkroom for a period of half an hour. When the containers were again returned to diffuse daylight, the larvae showed no change in their reaction to light. Neither was there any change in geotaxis. Similarly, larvae kept at reduced temperatures showed no change of tropisms, when they were returned to diffuse daylight after a 30-minute period in the darkroom.

On the other hand, there is some evidence that darkness reduces the activity of the larvae and decreases the length of the free-swimming period. Three experiments were performed by subjecting larvae to conditions that, in diffuse daylight, ordinarily prolong the natatory period well beyond 2 hours. Two vials, containing sea water diluted to a density of 1.010 grams/cc., were placed in the darkroom. When the first vial was inspected at the end of an hour, all the larvae were found to be active; but at the end of 2 hours only 28 per cent were active, whereas 55.8 per cent were still swimming in the controls. (For the effects of diluted sea water see Table V.) In the second vial none were active at the end of 2.5 hours, although 27.5 per cent of those in the controls were still motile at the end of 4 hours. The third vial contained normal sea water which was kept at a temperature reduced sufficiently to cause prolonged activity in diffuse daylight. None of the larvae in this vial were active at the end of an hour. Although more experiments are needed, the results indicate that darkness probably reduces the length of the natant period in this species.

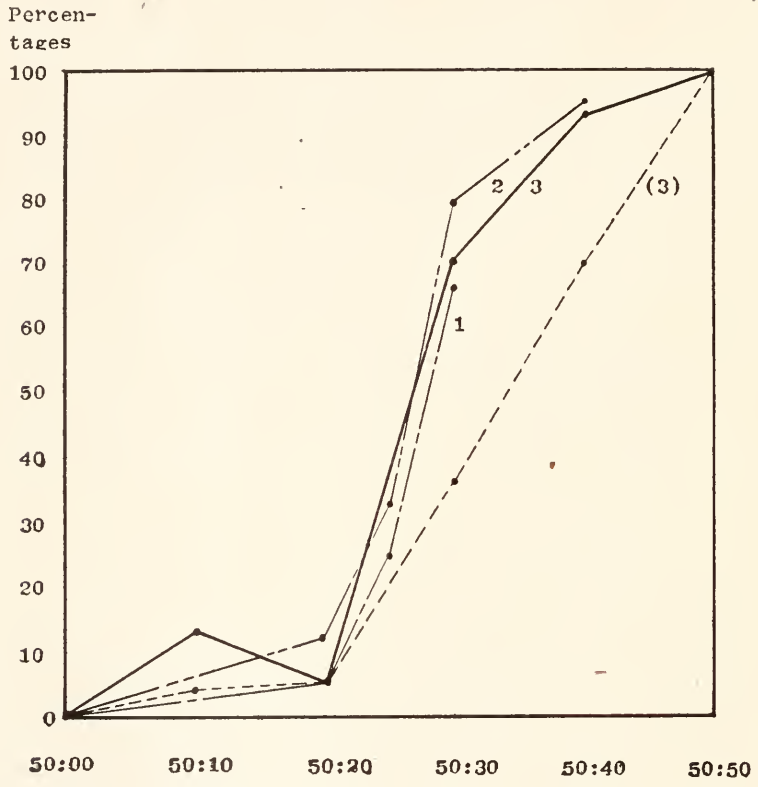
The effects of salinity on the duration of the free-swimming period and the responses of the larvae to light and gravity

Since *Bugula* grew profusely near the laboratory at the mouth of the Newport River, it was surprising that adult colonies could not be detected near the water line on pilings and docks only three-quarters of a mile up-stream. The few that were found were growing near the bottom of objects that could be raised to the surface. A sample of the water in this region, taken about an hour after the tide had turned and sea water had begun running up-stream, was found to have a specific gravity of 0.0116 (28° C.), whereas the lowest of 25 tests made on water near the laboratory gave a reading of 1.0164.[2] It seemed likely that this difference in density, although small, would not represent the maximum, since at low tide the amount of fresh water would undoubtedly be greater and the density would be correspondingly lower.

That the salt content of the water might affect the geotropic responses of the larvae and determine their ultimate place of attachment seemed to be the only explanation for the observed distribution. Furthermore, on several occasions during the summer of 1944 the writer had noticed that larvae seemed to metamorphose in an abnormally short time when concentrated sea water was added to a well-slide. Consequently, a series of experiments were performed by using various concentrations of sea water below and above the normal range. Since 16 out of 25 samples of sea water in the region of the laboratory showed a specific gravity of 1.0200–

[2] In order to avoid unwieldly decimals in Table V, temperature corrections were not made. The maximum deviations due to temperature fluctuations would not exceed 0.00076 gr./cc.

1.0226, this may be considered approximately the normal range during the time the experiments were performed. These samples were taken on different days at both high and low tide during a three-week period. The highest reading was 1.0226 and the lowest was 1.0154; seven readings showed a prevailing density of 1.0164–1.0173 gr./cc. during a period of especially heavy rainfall in the third week of August. Gutsell (1930), however, found a higher average density during the



Graph I. Dilutions of sea water (50 cc.) with distilled water

Lines 1 and 3 (solid) show the percentages of geopositive larvae at the end of 1.5 hrs. Line 2 (solid) shows the number geopositive at 5.5 hrs. Dotted line (3) shows the number active at the end of 1.5 hrs. in vials used for solid line 3.

years his readings were taken (1924–28), even if temperature corrections are made for those listed above; at that time the salinity in this region approximated 30 parts per mille, but occasionally ran as high as 38 or as low as 6 parts per mille (20° C.).

Graph I shows the results of 11 experiments carried out by diluting 50 cc. of sea water with 10 to 50 parts of distilled water. The temperatures were read at the time the densities were taken, and the observations were carried out on the same day between 11 A.M. and 4:30 P.M. (E.S.T.). The following table shows the densities in gr./cc. of the various dilutions used. (The writer found that the

density of a sample of sea water rose approximately 0.00017 gr./cc. for each degree (C.) drop in temperature.) It will be noted that dilutions of 50:10 and 50:20 had but little effect on the number of larvae that became geopositive. The number of geopositive larvae began to increase at dilutions greater than 50:20 and grew progressively larger as the salinity decreased. A decrease in salinity likewise increased the average length of the natatory period. Since the water three-quarters of a mile upstream had a density of 1.0116 gr./cc., a dilution slightly greater than 50:30, its low salt content may explain the location of *Bugula* colonies near the bottom of the stream.

TABLE IV

Dilution	Temperature	Gr./cc.
50:50	29.0	1.0101
50:40	29.5	1.0108
50:30	29.9	1.0119
50:20	30.0	1.0149
50:10	30.0	1.0169
50:00	29.7	1.0228

Since random samples of the laboratory supply of sea water, taken from the mouth of the Newport River, showed fluctuations caused not only by high and low tide but also by the amount of rainfall, more extensive data were obtained by using sea water diluted or concentrated to a definite specific gravity as determined by means of a very sensitive hydrometer. Care was taken to fill the vials to the same level (about 5.5 cm.) and to add approximately the same amount of water, when larvae were pipetted from the finger-bowls. The addition of such small amounts of water to the vials did not seriously increase the densities in the lower ranges, where experimental results were most pronounced. The introduction of non-motile larvae, which cannot respond to treatment, was avoided by first transferring larvae from the finger-bowls to a stender dish of fresh sea water before introducing them into the vials. Since the active ones invariably swam to the lighted side of the dish within a minute or two, large numbers could be transferred without difficulty. Because quiescent larvae sometimes become active after a few minutes of observation, counts had to be made 3 or 4 times for each vial. The difficulty of determining the number of larvae that were active at a given time necessitated the use of only small populations for each vial; the ideal number was found to be about 20 to 30. For these experiments 2,358 larvae were counted during a 5-week period. The results are summarized in Table V.

Larvae kept in water having a specific gravity of 1.010 to 1.012 were extremely active for an abnormally long time, definitely photopositive during the time of greatest activity and geonegative during the early stages of the natatory period. The majority, however, descended to the bottom towards the end of the free-swimming period. These larvae were visibly smaller than those that had a shorter period of activity and measured only 0.14 mm. in diameter as contrasted with the average size, 0.20–0.22 × 0.27–0.30 mm. Since this decrease in volume was found to be correlated with a prolongation of the free-swimming period, it seems likely that the activity of the larvae resulted in a conversion of stored food material into

waste products of metabolism that passed out into the surrounding medium. It is not improbable that the lipoid granules observed by Barrois (1877) and others constitute a reserve food supply for those forms of bryozoan larvae without an alimentary tract. Furthermore, these larvae had extruded an orange colored pigment that gave a characteristic color to the water in the bottom of the vials.

TABLE V

Specific gravity	Number of larvae	Percentage geonegative in hours				Percentage intermediate				Percentage geopositive				Percentage active					
		½	1	2	4	½	1	2	4	½	1	2	4	½	1	2	4	Number larvae	6
1.010	203	44	30	8	6	18	28	38	6	38	42	54	88	100	87	67	39	44	16
1.011	126	72	42	34	22	8	32	36	10	20	26	30	68	97	88	52	38	26	8
1.012	135	85	76	77	65	5	6	6	5	10	18	17	30	87	55	38	27	39	10
1.013	207	98	92	83	58	0	0	0	7	2	8	17	35	59	55	30	9	54	5
1.014	85	96	94	93	86	3	2	2	1	1	4	5	13	66	26	10	7	43	9
1.015	137	88	89	90	93	11	4	4	0	1	7	6	7	65	31	4	1	44	0
1.016	168	84	87	90	90	7	4	1	1	9	9	9	9	28	10	3	0	96	0
1.017	143	88	89	89	90	2	2	2	1	10	9	9	9	26	9	4	2	187	3
1.018	112	92	92	93	93	1	1	0	1	7	7	7	6	32	7	2	1	32	0
1.019	148	98	97	97	97	0	0	0	0	2	3	3	3	12	2	0	0	85	0
1.020	159	95	98	96	96	2	1	1	0	3	1	3	4	21	6	1	1	26	0
1.021	171	97	90	94	93	1	3	3	2	2	7	3	5	19	5	3	1	95	2
1.022	97	95	—	—	94	0	—	—	0	5	—	—	6	10	5	0	0	63	0
1.023	104	83	85	86	88	10	5	5	3	7	10	9	9	25	5	2	0	62	0
1.024	115	69	66	65	65	11	11	12	13	20	23	23	22	30	9	1	0	43	0
1.025	214	15	16	16	16	12	10	11	11	73	74	73	73	8	3	1	0	57	0
1.026	134	7	5	6	6	6	10	7	4	87	85	87	90	13	9	3	2	39	0
Total	2358																		

Temperature of sea water for above—25–29°C.
Temperature at which hydrometer readings were taken—27.5–29°C.

The larvae themselves were orange-brown, whereas those kept in water of higher specific gravities always appeared black. Lillie (1909) found a similar pigment in the larvae of *Arenicola;* this, he said, is derived from the egg cell and normally disappears during later larval stages. Prolonged muscular activity caused this pigment to leave the cells and color the surrounding medium with a faint yellow tinge.

Larvae placed in sea water at densities between 1.016 and 1.022 gr./cc. (the normal range) behaved quite differently from those in the lower densities. An average of 92.1 per cent remained geonegative at the end of 4 hours, and 90 per cent at the time of fixation; in vials containing sea water at a density of 1.010 gr./cc., however, only 6 per cent were geonegative at the end of 4 hours and at the end of the free-swimming period. Furthermore, those kept in sea water at densities between 1.016 and 1.022 gr./cc. had considerably abbreviated natatory periods when contrasted with larvae in water at lower densities. At the end of 4 hours the average percentage of active larvae in 44 vials containing water at the higher densities (1.016–1.022) was 0.71 per cent whereas 34 per cent were active at densities from 1.010 to 1.012 gr./cc. inclusive.

In sea water more concentrated than 1.022 gr./cc. the percentage of larvae that descended to the bottom increased with the density. In these cases, however, a geopositive reaction was not preceded by a prolonged period of activity. At densities of 1.025 and 1.026 gr./cc. only 10.5 per cent were active at the end of half an hour, whereas 22.5 per cent were active in concentrations between 1.016 and 1.024 gr./cc. and 100 per cent were active at a density of 1.010 gr./cc. At a density of 1.026 gr./cc. only a few larvae were active after 10 minutes; they sank passively to the bottom almost as soon as they were introduced into the vials, and generally only feeble movements could be detected afterwards. Evidently the factors that caused these larvae to descend to the bottom were quite different from those that brought about a similar positive geotaxis in sea water of low salt concentration. In the latter, the larvae swam slowly to the bottom after they had been active a very long time. When the effects of concentrated sea water were observed under the microscope, it was found that larvae metamorphosed in a remarkably short time in almost epidemic proportions. It seems unlikely that the organisms were effected by any change in the pH produced by diluting or concentrating the medium, for subsequent tests showed that a dilution to 1.010 gr./cc. caused the pH to drop only 0.08 and a concentration to 1.026 increased the pH only 0.52 ± 0.1.

The development of larvae at various salinities

Vials containing larvae that had already formed zoöids were inspected at the end of 24 hours. Those in more concentrated sea water were slightly larger than normal. All of them had their tentacles everted and snapping actively at this time. These zoöids were attached securely to the substrate.

Those in vials containing sea water at specific gravities of 1.016 and 1.017 were normal in every respect at 29 hours, except that they did not withdraw their tentacles as a reaction to mechanical jarring, as they normally do in higher salt concentrations. Those that developed at a density of 1.015 gr./cc. had their tentacles everted, but these were protruding at an angle of 90° to the normal position. Apparently these organisms were entirely incapable of withdrawing them, for mechanical jarring had no effect during the 30 minutes they were under observation.

In vials containing sea water at densities below 1.015 gr./cc. development was poor. There was a marked reduction in size, and none had their tentacles everted (29 hours). Nevertheless, even those that metamorphosed in water having a density of 1.010 gr./cc. had normally formed internal organs that moved spasmodically at times, and most of them had a bud for the formation of a second zoöid. Again it seems likely that the poor development of these zoöids can be attributed to an insufficiency of stored food at the time of metamorphosis, caused by their prolonged activity. Apparently some of the larvae in water at a density of 1.010 had failed to metamorphose; and many of them did not adhere to the bottoms of the vials, for they could be easily dislodged by a stream of water.

Salinity vs. osmotic pressure

In order to determine whether the observed effects were due to the salt content or to the osmotic pressure, sea water was diluted to a density of 1.010 gr./cc. and sucrose was added until the specific gravity reached 1.026. Since larvae in sea water at a density of 1.010 gr./cc. remained active for an unusually long time, it was considered logical to conclude that osmotic pressure would not be a factor, if the

larvae showed the same behavior after the pressure had been raised by sucrose. On the other hand, if an abbreviation of the natatory period took place, it could be concluded that this effect was caused by an increase in osmotic pressure.

Since subsequent tests showed that similar sucrose solutions had a freezing point depression equal to that of sea water at a density of 1.014 (see Table VI), vials con-

TABLE VI

Density in gr./cc. of original sea water	Substance added	Final density gr./cc.	Freezing point depression	pH
1.010	NaCl	1.017	1.74	7.6 ±.10
1.010	$CaCl_2$	1.017	1.36±.02	7.07±.07
1.010	KCl	1.017	1.67±.11	7.61±.05
1.010	$MgC._2$	1.017		7.58±.14
1.020	$CaCl_2$	1.026	2.20	7.85
1.010	Sucrose	1.026	1.25±.02	7.2 ±.20
1.010	Sucrose	1.017	—	6.7
1.010	Glucose	1.026	1.41	6.2
Distilled	NaCl	1.026	—	6.3
1.017	None	1.017	1.51±.07	7.67±.02
1.024	None	1.024	2.06±.03	8.25
1.026	None	1.026	2.21±.07	8.25±.05
1.010	None	1.010	.96	7.60

± indicate repeated experiments.

Osmotic pressures of sea water at various concentrations. Temperature = 27.5–29.0° C.

Specific gravity	Freezing point depression	Pressure in atmospheres
1.010	0.96	11.56
1.011	1.09	13.12
1.012	1.11	13.36
1.013	1.17	14.08
1.014	1.26	15.16
1.015	1.36±.03	15.36±.36
1.016	1.46±.07	17.56±.84
1.017	1.51±.07	18.16±.84
1.018	1.62±.01	19.48±.12
1.019	1.62±.01	19.48±.12
1.020	1.67±.05	20.08±.60
1.021	1.87	22.48
1.022	1.87	22.48
1.023	1.95	23.44
1.024	2.06±.03	24.75±.35
1.025	2.10±.02	25.23±.25
1.026	2.21±.07	26.55±.96

± indicate repeated experiments.

taining water at this density may be considered controls. In the sucrose solutions 45.3 per cent of the larvae were active at the end of 4 hours and 94 per cent had become geopositive; in the controls only 6.9 per cent were active and 13 per cent had descended to the bottom. It seems apparent that an increase in osmotic pressure by a non-electrolyte does not have the same effect as the addition of ions normally pres-

ent in sea water. These results are in agreement with the observations of Loeb (1900), for he found that marine organisms are easily affected by a disturbance in ionic balance, but are practically independent of osmotic pressure.

Larvae placed in a similar solution of glucose and sea water behaved quite differently, for they immediately sank to the bottom and remained immobile. When the pH values were obtained for similar solutions made with the same glucose, it was found that they were somewhat acid when freshly made (5.8–6.2); by the end of 24 hours the pH had fallen to 4.5, and by 48 hours had reached 3.4. Since the solutions used at Beaufort were made the day before the experiments were performed, presumably the anomalous behavior of the larvae can be attributed to an originally high degree of acidity, which was further enhanced by bacterial action. The sucrose solutions, however, showed no great acidity, for they dropped to only 6.5 at the end of 24 hours. The effect of pH on the behavior of other species of larvae investigated recently at Woods Hole will be described in another paper.

The effects of various ions on the length of the free-swimming period and the metamorphosis of the larvae

Since the experiments just described gave presumptive evidence that variations in salt content played a far more important role than an accompanying increase or decrease in osmotic pressure, the following observations were made to determine the specific effects of the four ions most abundant in sea water by using the chlorides of the metals. For comparing the effects of normal sea water with sea water having a high concentration of only one of the ions, a "fundamental solution" was made by diluting sea water to a specific gravity of 1.010. Since this concentration contains ions in the same proportion as normal sea water and is capable of supporting prolonged larval life, it may be presumed that any modification of the behavior of larvae can be attributed to the ions that were added. The osmotic pressures and pH values of similar solutions are given in Table VI.[3]

Effects of sodium chloride. Larvae placed in pure solutions, made by adding sodium chloride to distilled water until a specific gravity of 1.026 was obtained, sank to the bottom immediately, became immobile and turned completely white within three minutes, so that they could be seen easily only against a black background. When viewed under the microscope, the larvae were observed to undergo metamorphosis very rapidly. Their white appearance was due largely to the enormous amount of milky white holdfast material ejected and to an inward migration of pigment granules from the surface of the larvae. After the initial stage of metamorphosis had begun, the succeeding stages occurred somewhat more slowly than in normal sea water. Most of the larvae were shrunken and appeared to be about two-thirds normal volume. At the end of 3 hours they looked like irregular pieces of white jelly; their edges were ragged and protoplasmic threads protruded from their surfaces. Those on the bottom of the vials were not anchored securely and could be easily disloged by directing water from a pipette against them; and, after being dislodged, they floated to the top. Evidently metamorphosis had decreased their density. Similar results were obtained with solutions made by adding enough sodium chloride to distilled water to raise the specific gravity to only 1.010, but in this case

[3] Densities were measured in connection with ion effects because equipment was easily available for such measurements and these effects were to be compared with other results in which density measurements had been used.

the larvae did not take on a characteristic white appearance until 30 minutes had elapsed. Metamorphosis, therefore, did not occur so rapidly.

Finally a "fundamental solution" was made and raised to a specific gravity of 1.017 by sodium chloride. When first admitted to the vials, the larvae sank to the bottom immediately; but after a few minutes almost all had ascended to the top and were swimming actively. At the end of an hour 16 per cent were still active and 72 per cent had descended to the bottom and remained there, whereas in normal sea water of the same osmotic pressure only 5 per cent were active and 3 per cent were geopositive. The first reaction of larvae placed in a new environment seems to be what Marcus (1926a) called a "schreckreaktion"; they sink down passively and remain motionless for a few minutes. This reaction, when observed under the microscope, was found to be preceded by a violent constriction of the organisms in the region of the equator. Similar behavior was reported for the nauplii of *Balanus amphitrite* by Edmondson and Ingram (1939).

Effects of potassium chloride. The first solution was made hypertonic to the medium from which the larvae were taken by adding potassium chloride to normal sea water (density, 1.020 gr./cc.) until a specific gravity of 1.026 was obtained. The second was made isosmotic to sea water having a density of 1.020 by adding the chloride to a "fundamental solution." In both solutions the larvae sank immediately to the bottom and became milky white as they left a trail of black pigment granules behind them. After reaching the bottom, a few remained slightly active for about 10 minutes. They were somewhat more active in the second solution of lesser density, moving slowly and aimlessly on the bottom, although the cilia were beating in an almost normal manner. Perhaps the effective stroke was reduced enough to prevent active movement. After an hour their appearance was similar to those in the pure solutions of sodium chloride, except that metamorphosis did not occur. Potassium chloride had a similar effect in causing the loss of pigment from already metamorphosed larvae that were floating on the surface of finger-bowls, but in this case the pigment streamed out below the larvae and sank to the bottom; the larvae themselves remained on the surface. Lillie (1909) observed a similar loss of pigment in *Arenicola* larvae placed in potassium solutions (cf., also, Chambers and Reznikoff, 1926).

Effects of calcium. Larvae placed in distilled water raised to a specific gravity of 1.010 by calcium chloride went to the bottom and ceased movement abruptly. At the end of an hour, they looked orange-brown and flattened like oblate spheroids, caused by a shortening of the oral-apical axis; they closely resembled larvae that had a prolonged natatory period in sea water reduced to a density of 1.010 gr./cc., except that those in the calcium chloride solution were larger than normal, measuring 0.29–0.30 × 0.30–0.34 mm. When other larvae were placed in a solution made hypertonic to the medium from which they were taken (sea water at a specific gravity of 1.020) by the addition of calcium chloride until a density of 1.026 gr./cc. was reached, they likewise sank to the bottom but remained slightly active for 2 hours. The cilia and vibratile flagella continued to beat rapidly, even though the larvae themselves did not move. Although the holdfasts had been ejected in many of them, normal attachment did not take place and the larvae continued to move aimlessly around with their holdfasts trailing behind them. They were badly fragmented and considerably reduced in size, measuring only 0.18–0.19 mm. along their

longer diameter. Finally, calcium was added to the "fundamental solution" to bring the specific gravity up to 1.017. Larvae in this solution showed a marked prolongation of the free-swimming period. In one vial 20 per cent were still active after 8 hours, In another, 2 larvae were still active after 12 hours, a strikingly long time in comparison with the normal period of 30–60 minutes. These larvae, in contrast to those in isosmotic sea water (specific gravity, 1.015), behaved quite differently from the latter. Not only did they have a decidedly longer natatory stage, but all, without exception, became geopositive at the end of the free-swimming period; only 8 per cent of those in isosmotic sea water became similarly geopositive. These larvae in the calcium chloride solutions behaved like those in normal sea water diluted to a density of 1.010 gr./cc. Like the latter, they remained geonegative, swimming just under the surface, during the greater part of their larval period, before they descended to the bottom. There was one significant difference, however, for they remained active much longer than those in diluted sea water. Furthermore, they did not attach rigidly like the latter.

Magnesium chloride. Sea water diluted to a density of 1.010 gr./cc. was raised to a specific gravity of 1.017 by magnesium chloride, so as to approximate the osmotic pressure of normal sea water. Larvae in this solution showed an initial shock reaction and sank to the bottom. They recovered rapidly, however, swam to the top and continued to swim feebly at the surface for half an hour. They showed a pronounced tendency to coalesce in groups. Lillie (1909), who observed a similar coalescence of the larvae of *Arenicola* in high concentrations of magnesium chloride, attributed this behavior to a loss of muscular contractility without an accompanying decrease in ciliary movement. By half an hour, active swimming movements had ceased. Whenever larvae at the surface became quiescent, jarring the vials caused them to become active again. Each time this was repeated, several larvae swam downward, after a brief period of swimming at the top, and remained on the bottom. By the end of 40 minutes, all the larvae had descended to the bottoms of the vials. Although they were not swimming, these larvae lying inert on the bottom had failed to attach and metamorphose. Five hours and 45 minutes after they were placed in these solutions, pipettes-full of larvae were removed and added to normal sea water; the majority began to swim actively again.

Mechanical agitation

Although the vertical distribution observed by McDougall (1943) could not be attributed to modifications of any of the factors thus far investigated, since they had to be more extreme than any prevailing in nature, nevertheless one correlation became increasingly evident. Prolonged activity of the larvae was always associated with a positive geotaxis. This was true, whether induced by a reduction of temperature or salinity or by an excess of calcium over the other ions present in sea water. Furthermore, mechanical jarring, by activating the larvae in solutions of magnesium chloride, had caused them to descend to the bottom. Agitation by waves and wind seemed to be the stimulus provided by nature for bringing about a positive geotaxis. Moreover, experiments on other animals have shown a correlation between mechanical agitation and a change in physiological state. Rogick (1939), for instance, found that excessive handling of the larvae of fresh-water bryozoans abnormally prolonged and interfered with metamorphosis. Similarly, contact

stimuli frequently change the phototropism of organisms, as evidenced by the experiments of Holmes (1905) on *Ranatra*, Parker (1902) on *Labidocera* and Towle (1920) on *Cypridopsis*. The writer likewise observed that the larvae of *B. neritina* became negatively phototropic for about a minute after they were ejected from a pipette.

To determine the effect of mechanical agitation on larval behavior, an air jet from a pipette was directed against the surface of the water whenever larvae showed a tendency to become quiescent. This caused them to swim actively again. In brief, 19 per cent were active and 62.5 per cent were geopositive in the experimental vial at the end of 4 hours, whereas only 11 per cent were active and 11 per cent were geopositive in the control. In view of the correlation that exists between mechanical agitation and swimming movements on the one hand and between prolonged activity and a positive geotaxis on the other, it seems likely that further experiments would yield similar or even better results. Since the writer never observed that larvae were liberated in darkness under laboratory conditions, it is difficult to explain the observation that in nature they attach as readily at night as in the daytime (Edmondson and Ingram, 1939; Edmondson, 1944), unless it is assumed that these organisms, liberated by light, remain active through mechanical agitation during the day and part of the night. Certain experiments performed by the writer indicate that light, as well as mechanical agitation, may serve as a stimulus to movement.

Discussion

The nature of geotaxis in B. neritina

Mechanical agitation is one of the factors existing in nature that is not generally duplicated under laboratory conditions, even though all the other factors belonging to the habitat of an organism seem to be present. The abnormally tranquil surfaces prevailing in the laboratory are not at all comparable to those encountered by larvae when they reach the surface of the ocean and are constantly buffeted about by waves and wind. Presumably larvae in their natural surroundings have a somewhat longer free-swimming period than those under laboratory conditions. The action of waves and wind prevents them from attaining that degree of quiescence which is a natural prerequisite for metamorphosis. Thus they remain active; and, as a result of prolonged activity, they descend to regions where a greater degree of calmness prevails. Crevices in rocks and pilings undoubtedly afford a certain amount of protection against the buffeting action of waves; hence a natural explanation may be given for the preponderance of young zoöids that can be observed in grooves and in holes bored into test panels, after they have been submerged for a few days. At the present time there cannot be given a completely empirical explanation that will clearly demonstrate the *mŏdus operandi* of the various factors that bring about a positive geotaxis under natural conditions.

Geotaxis in marine larvae cannot be assigned to the same category of responses as the other tropisms. Phototropism, for instance, whether it be positive or negative, is an active kind of response. On the other hand, what we call a positive or negative geotaxis can be resolved into other mechanisms. A negative geotaxis can be attributed to ciliary action, to buoyancy or to a more abundant supply of oxygen near the surface. It has sometimes been identified with a positive phototropism. Many aquatic larvae, such as those of *Arenicola* (Lillie, 1901) or of the phylacto-

laematous Bryozoa (Marcus, 1926c), are specifically denser than the surrounding medium. They can keep afloat only by ciliary movement, and when the cilia are injured they sink to the bottom. Others, apparently, are lighter than the surrounding medium and are buoyed up passively by Archimedes' Principle. Hora (1930) stated that some organisms can decrease their specific gravity by a reduction of the abdominal cavity; others, such as the larvae of *Megalophrys,* have hydrostatic organs. Thus their rising or sinking depends upon their density. Similarly, Visscher (1928) observed that the cyprid larvae of *Balanus eburneus* have a fatty substance in the anterior of their bodies that acts like a buoy in holding them near the surface; this lipoid material gradually disappears towards the end of the pelagic period. McDougall (1943) found that the setting of these larvae occurred in greatest abundance at a point some 5 or 6 feet below the surface. In another species, *Chthamalus fragilis,* however, he found that the lipoid droplets do not disappear towards the end of the free-swimming period; the highest incidence of settings of this species was very near the surface, and no attached larvae were found more than 6 inches below mean low water. Evidently buoyancy, or the lack of it, can determine the vertical distribution of certain organisms. In the cyprid larvae just described phototropism is not a factor in determining the ultimate place of fixation, for Visscher (1928) found that both species are photonegative just before attachment. A positive geotaxis should not be confused with a negative phototropism, as has been done sometimes in the case of bryozoan larvae. Sometimes, at least, the two tropisms are dependent upon entirely different mechanisms.

There are several possible explanations for differences in the behavior of the larvae of *B. neritina* in warm and cold water. It might be assumed that, since oxygen would be more abundant near the surface, the larvae would collect there when the temperature is high, because they are unable to obtain sufficient oxygen to support their increased metabolic activity, if they venture far from the surface film. The possibility that a negative geotaxis may be simply a positive response to oxygen in the case of free-swimming larvae has been suggested by Marcus (1926a) as a plausible explanation for the pelagic habits of certain marine Bryozoa. In his experiments on fresh-water Bryozoa (1926c), however, he could not obtain conclusive evidence that the larvae move in the direction of an increasing oxygen gradient. The writer, also, found no confirmation of such a theory. When a vial of sea water was inverted over a petri dish with the bottom supported on two slides so that oxygen could enter only from below, the larvae showed not the slightest tendency to move downward. Neither did they surround an air bubble that was on the side of the vial opposite the source of illumination. Furthermore, it would be difficult to explain a positive response to gravity, when the temperature is lowered, on the assumption that the negative geotaxis exhibited in warm water is merely a positive response to oxygen. Even though the oxygen content of water at reduced temperatures might be sufficient to maintain the lowered metabolic requirements of larvae at various depths beneath the surface, their movement in a direction of a slightly decreasing oxygen gradient would require explanation.

At first sight, it would seem logical to attribute the rising or sinking of the larvae to ciliary action alone, if it is assumed that these organisms are denser than the surrounding medium. Their descent in hypotonic solutions only after prolonged swimming might be attributable to ciliary fatigue. Since a reduction in

temperature decreases ciliary movement in the gills of *Mytilus* (Gray, 1923) and since hypertonic solutions (Gray, 1922), sea water having a pH below 5.5–6.0 (Gray, 1920) and solutions of sodium and potassium (Gray, 1920) have an even more deleterious effect, the sinking of the larvae of *B. neritina* under almost identical conditions might seem to be due solely to attenuation of ciliary action. The prolonged negative geotaxis of these organisms in solutions containing calcium concentrated to a certain optimum would seem to be correlated with the favorable effects of this ion on ciliary action, as described by Lillie (1901) for *Arenicola*. On the other hand, it might be expected that ciliary action would be maintained better in solutions containing an excess of potassium ions than in a similar sodium solution of approximately the same pH and osmotic pressure, for the latter has a more pronounced inhibitory effect on the cilia of *Mytilus* (Gray, 1920) or of veliger (Mayer, 1911) or *Arenicola* (Lillie, 1901) larvae.

The larvae of *B. neritina*, although they swim sluggishly in a horizontal plane just beneath the surface film in warm water, give no indication that they remain there only by active swimming. In fact, both dead and metamorphosed larvae were always found floating at the surface, and obviously ciliary action cannot account for this. They seem rather to be buoyed up by the medium. If this is true, they would differ from the phylactolaematous Bryozoa, which maintain themselves at the surface only by active swimming and sink downwards when injured by heat or by other unfavorable conditions (Marcus, 1926c). Again, larvae swam without exception towards the side of a vessel nearer the source of diffuse daylight, whereas only 42.2 per cent swam downward when they were illuminated only from below. This difference in behavior would seem to indicate that ciliary action is much more effective in moving them along a horizontal plane than vertically downwards against what appears to be a buoyancy gradient. Presumably the orientation of their long axes determines their direction of motion. It cannot be assumed, however, that larvae at room temperature do not swim beneath the surface because their apical ends are directed upwards in orientation to light entering obliquely from above, for they remain on the surface in darkness. If the larvae are heavier than the medium, it should be easier for them to swim downward, for the pull of gravity would be added to their ciliary action; consequently, it would be logical to expect that large numbers of larvae kept in darkness and unoriented to light would swim downward and that this response would be universal in the case of larvae illuminated only from below.

A reduction of ciliary action alone does not seem to be an adequate explanation for a positive geotaxis in these larvae; it seems necessary to posit a simultaneous increase in density. Since larvae invariably became geopositive after they had been active for an abnormally long time, it seems probable that their descent to the bottom can be attributed to a hydrostatic principle. The vitelline mass within these organisms may decrease gradually in volume as they grow older and swim longer; and since it is composed largely of fatty globules of low specific gravity, depletion of lipoids and shrinkage of the larvae would decrease their volume and increase their density, thus causing them to descend at the end of the pelagic stage. This view is confirmed by the observation that larvae smaller than average descended to the bottom, whether this condition was attributable merely to normal variations in size or to a visible reduction in volume resulting from an experimentally induced pro-

longation of activity. After prolonged swimming at the surface, larvae were noticeably smaller when they descended to the bottom in solutions of glucose, calcium and hypotonic sea water. In the latter solution it seems probable that they would have swollen somewhat in the beginning and would, therefore, have displaced a greater volume of the medium. They would thus be buoyed up until their volume decreased again through a conversion of food into waste products of metabolism. Since larvae sank passively in hypertonic sea water, they probably lost fluid to the medium, and their subsequent reduction in volume may have made them denser than the solution. Their rapid descent, however, apparently cannot be explained on the principle of osmosis alone. Probably both injury to the cilia and a violent muscular contraction were involved. An initial shock reaction was actually observed under the microscope in larvae exposed to an excess of certain ions; they underwent a violent constriction in the equatorial region, which may have decreased their volume without altering their weight appreciably.

Since du Bois-Reymond (1914) has shown that the same temperature increment will cause a piece of tissue to expand three times as much as a similar volume of water, it seems likely that larvae may sink to the bottom in cold water in a comparatively short time (15–20 min.) because of an increase in density brought about by shrinkage. Similarly the failure of larvae to descend in warm water might be attributed to a swelling of the vitelline mass, which would cause them to displace a greater volume of water; buoyancy would thus hold them just beneath the surface film. Their reaction recalls the behavior of orthotoluidine drops that float on the surface of warm water, but sink to the bottom when the temperature is reduced; like the larvae, these drops, having approximately the same density as water, will rise again when the medium is warmed only a few degrees. What seems to be a downward spiralling movement of the larvae may be merely a succession of circular paths at descending horizontal levels.

Factors influencing metamorphosis

Only conjectures may be offered at this time regarding the effects of various solutions on the onset of metamorphosis. At first, the writer was inclined to assume that sodium itself is a specific agent that brings about metamorphois. The rapid setting of larvae in sodium solutions would suggest this hypothesis. Furthermore, sodium would presumably favor the violent muscular contraction necessary for the ejection of the holdfast. The fact that both calcium and magnesium either prevented metamorphosis or delayed it far beyond the normal time of onset might be interpreted on the basis that these ions decreased the permeability of the larval tissues, so that sodium could enter only with difficulty. Concomitance of events, however, suggests but does not prove a causal relationship. In view of the fact that there seem to be many substances capable of inducing metamorphosis in ascidians (Berrill, 1930; Zinkin, 1938), it would not be illogical to predict that the same may be true of bryozoan larvae. If metamorphosis is essentially a process of dedifferentiation, as Huxley (1922) has suggested, it is quite possible that any inhibiting agent can bring it about. Copper is capable of inducing metamorphosis in the oyster (Prytherch, 1934) and has a similar role in ascidian larvae (Grave, 1941; Berthold and Mast, 1944). Miller (1946) reported that copper has some effect in hastening the onset of metamorphosis in bryozoan larvae, but he did not discuss the

experiments that led him to this conclusion. Consequently, it may be that sodium has no specific effect on the larvae of *B. neritina;* it may have either a direct effect by inhibition, and in this respect it would not be unique, or an indirect action by increasing the permeability of larval tissues to ions, such as copper, that have an oligodynamic action. (The effects of ions, especially sodium and copper, on two species from Woods Hole will be discussed more fully in a future paper.)

Caswell Grave (1936) postulated the existence of a by-product of metabolism in ascidian larvae, which is produced in greater quantity by energetic swimming movements. This later product, he says, unites with a "susceptibility factor" formed by the secretion of some larval tissue or organ in the production of a third substance, which apparently acts directly on the larval nerve centers to cause the progressive series of responses that constitute the process of metamorphosis. In *B. neritina*, however, it seems unlikely that the accumulation of metabolic waste products has any influence in hastening metamorphosis. *A posteriori,* it would be more logical to attribute the prolonged activity of larvae under certain conditions to factors that delay the accumulation or concentration of some substance that effects the onset of metamorphosis.

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Summary

1. The distribution, external morphology, swimming movements and metamorphosis of the larvae of *B. neritina* are discussed. During metamorphosis the eye-spots of the larva function in orientation to light.

2. At room temperature (26–30° C.) the larvae under laboratory conditions are geonegative and photopositive during the larval period. Just before metamorphosis they lose their intense positive reaction to light and become more or less indifferent.

3. A reduction of temperature causes larvae to become geopositive and simultaneously prolongs their free-swimming period. When either ascending or descending gradients of temperature are employed, the greater number of larvae change their responses to gravity between 20 and 23.5° C.

4. In cold water (7 to 10° C.) larvae lose their intense positive response to light and become more or less indifferent. Their behavior at reduced temperatures shows that they may become geopositive without exhibiting a simultaneous negative reaction to light. Their descent to the bottom of a vial is not caused by a negative phototropism. Either of the two tropisms may be independent of the other.

5. Larvae made geopositive by a reduction in temperature do not swim upwards towards a source of illumination, when the rays can enter a vial only from above. Neither do they react negatively to light, when the rays enter only from below. Their positive geotaxis cannot be modified by light. At room temperature, however, approximately half the larvae, originally geonegative, swim downwards towards a source of light placed beneath a vial.

6. At 28–30° C. the geotaxis of the larvae is not affected by rapid changes in light intensity.

7. At room temperature, intense light (sunlight and artificial illumination) has some effect in driving larvae to the bottom of a vial.

8. When larvae are returned to diffuse daylight after a 30-minute period in darkness, they do not change either their phototropism or their geotaxis, when they are maintained at low (7–8° C.) or high (26–30° C.) temperatures. Darkness, however, probably reduces their activity and shortens the larval period.

9. A reduction (40–50 per cent) of the salt content of sea water greatly prolongs the natatory period and causes larvae to become geonegative after a long period of swimming at the surface. Hypertonic sea water, however, greatly reduces the free-swimming period, induces a more rapid onset of metamorphosis and causes the larvae to become geopositive. The development of larvae that metamorphosed in sea water of various salinities is discussed.

10. A slight increase in the osmotic pressure of diluted (50 per cent) sea water by the addition of a non-electrolyte does not cause the same response as a similar increase in salt content.

11. An excess of either sodium or potassium causes a rapid loss of pigment. Potassium has the more pronounced effect. Sodium induces a rapid onset of metamorphosis, but potassium does not. Both sodium and potassium cause rapid sedimentation of larvae.

12. A similar excess of calcium greatly prolongs swimming movements and inhibits metamorphosis. An excess of magnesium inhibits metamorphosis, but does not cause prolonged swimming.

13. Mechanical agitation, hypotonic sea water and calcium apparently bring about a positive geotaxis by abnormally prolonging the free-swimming period of the larvae.

14. A tentative explanation of geotaxis in these organisms and a discussion of the effects of various factors on their metamorphosis are presented.

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VARIATIONS OF THE SUBMICROSCOPIC STRUCTURE OF THE CORTICAL LAYER OF FERTILIZED AND PARTHENOGENETIC SEA URCHIN EGGS

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Introduction

In previous papers one of us (A. Monroy and A. Monroy Oddo, 1945, 1946) has investigated by means of polarized light the submicroscopic structure of the cortical layer of the unfertilized sea urchin egg. The cortical layer shows positive birefringence and normal optic axis, and since, in all the experiments made, no data came out suggesting that proteins enter into its constitution, Monroy and Monroy Oddo maintained that it is entirely of lipidic nature. According to its behavior in some experimental conditions, they considered it as a fluid crystal of the smectic type, which can be brought into the scheme of Bungenberg de Jong's complex ionic systems. However, according to Runnström and collaborators (1943–45), whose paper we were able to see only after the publication of Monroy and Monroy Oddo's note, the proteins also take part in the constitution of the cortical layer of both the unfertilized and fertilized sea urchin egg. This question will be discussed in a forthcoming paper.

We deal here with some results obtained by studying the variations of the submicroscopic structure of the cortical layer of the fertilized or parthenogenetically activated egg. Monroy (1945) has already pointed out some rhythmical variations of the cortical birefringence after fertilization, up to the two blastomere stage. We thought it worthwhile to investigate more accurately the rhythms of the observed variations, putting them in relation with the process of mitosis and with the modifications of permeability which can be observed in the fertilized and parthenogenetic egg.

A short note on these findings has already been published in *Nature* (Aug. 17, 1946).

Experiments on Fertilized and Parthenogenetically Activated Eggs

Observations and experiments were carried on in February–April, 1946, at the Zoological Station, Naples, on eggs of *Psammechinus miliaris* artificially fertilized or activated by Loeb's method. For this purpose, the unfertilized eggs were treated with a solution of 2.8–3.2 cc. of N/10 butyric acid in 50 cc. sea water. After washing in slightly alkaline sea water, they were observed in pure sea water.

During the first series of experiments the temperature of the water was 13°–15° C., in the second series (April), 17°–19°. The developmental cycle was of course remarkably accelerated by the rise of temperature: in the first series the first cleavage of the fertilized eggs and the climax of the first monastral cycle in the parthenogenetic eggs took place after 1 hour and 30 minutes, in the second series after one hour.

Birefringence of the cortical layer

Fertilized eggs. At fertilization the cortical birefringence of the unfertilized egg disappears, while in polarized light the egg surface acquires a slight diffuse luminosity (Fig. 3). After ca. 15–20 minutes, when the sperm-aster is in course of development, a slight silver-white positive birefringence appears in the cortical layer. In a short time its intensity increases (although it never reaches the intensity it has in the unfertilized egg, and remains always white) and later on it decreases and disappears before the total regression of the sperm-aster. From the very first series of experiments, we noticed a somewhat irregular behavior of this first birefringence cycle; its relation to the sperm-aster cycle was not constant, in some cases its appearance being earlier, in others later than the beginning of the sperm-aster.

In the second series of experiments, which we performed at a higher temperature, the first birefringence cycle was even more irregular. In some lots we could not see it, in others it appeared irregularly and for a short time only in some of the eggs, usually before the sperm-aster was visible. We shall discuss the possible meaning of this behavior later, in connection with the experiments with hypertonic solutions.

A second cycle of cortical birefringence, also with positive character, extends from the metaphase to the telophase of the first mitosis, as already described by Monroy (1945). It is characterized by a much higher intensity of the birefringence, which sometimes reaches the same level as in the unfertilized egg. Furthermore, it is absolutely constant under our experimental conditions.

As soon as the first cleavage is completed, the cortical birefringence disappears. It appears again during the ana-telophase of the second mitotic cycle.

Parthenogenetic eggs. In the eggs kept in butyric acid solution, the birefringence becomes more or less white, decreases in intensity and sometimes disappears. After ca. 10 minutes, if the eggs are replaced in sea water, it resumes the intensity and the color of the unfertilized egg, and remains so indefinitely (observations up to one hour after treatment).

The eggs removed from the butyric acid solution after a few minutes and replaced in sea water are activated. A variable percentage, in the various lots, elevate the membrane, while other eggs show incomplete membranogenesis or a total failure of it, depending on individual differences between the females used and on slightly different concentrations or exposure times. The eggs with no membranogenesis, however, are also activated as appears from the nuclear and monastral cycles. It is known in fact, that in the eggs simply activated and not subjected to the second step of Loeb's treatment (hypertony), successive monastral cycles develop, which finally lead to cytolysis.

In the activated egg, with or without a fertilization membrane, the cortical birefringence disappears as in the fertilized eggs. From 15 to 20 minutes later the first birefringence cycle appears and its behavior is entirely similar to that of the fertilized eggs. Also in this case, if the eggs are kept at a higher temperature, the first cycle is more irregular.

The first cycle comes to its end, and, about one hour after activation, the second cycle begins, showing the same characters of constancy and intensity as in fertilized eggs. When the monaster regresses, the cortical birefringence also fades away and

disappears. Half an hour later the second monaster cycle develops and the birefringence reappears. A third, fourth, fifth monaster cycle follows, with more and more accelerated rhythms, and the egg finally cytolyzes. We observed the reappearance of the birefringence up to the third monaster cycle, and we have little doubt that it appears also in the following.

To summarize, the observations of fertilized and parthenogenetic eggs in polarized light show that in both cases there exists a first cycle of cortical birefringence, rather labile, appearing ca. 15–20 minutes after fertilization or parthenogenetic activation. It soon fades away and the egg shows a slight diffuse luminosity, which is probably due to small granules. Almost synchronic with the maximum expansion of the diaster, or the monaster, the cortical birefringence reappears very intensely. This second cycle also fades away, and a third appears at the metaphase of the second mitosis or at the expansion of the second monaster in parthenogenesis. The birefringent layer is always immediately underlying the hyaline coat of the activated egg.

Experiments with hypertony

The permeability of the unfertilized, fertilized and parthenogenetic egg has been the object of very many researches and from the experiments of various authors it appears that after activation the permeability undergoes a series of cyclic variations. We will not summarize all the pertinent literature, referring for that purpose to the papers by Herlant (1920), Hobson (1932), Runnström (1923, 1929), Öhman (1945). We will take into account mainly the results of the last two authors. They have shown that the fertilized or parthenogenetic eggs, after treatment with hypertonic saline solutions immediately after activation and for a period of about 10 minutes, manifest a uniform total contraction, preserving an almost unaltered spherical form. To this phenomenon Runnström has given the name of "kugelige Plasmolyse" or spherical plasmolysis. Between 10 and 40 minutes after activation the egg reacts in a different way to hypertonic solution, showing a wrinkled surface, that is, the "eckige Plasmolyse" of Runnström, or angular plasmolysis. After ca. 50 minutes from activation, the spherical plasmolysis reappears, and lasts until the moment immediately preceding the first cleavage when the angular plasmolysis sets in. Runnström (1929) thinks that such phenomena are related to variations of the gelification of the cortical layer, the angular plasmolysis corresponding to a condition of greater rigidity.

We thought that a certain correspondence might exist between the type of plasmolysis and cortical birefringence. Therefore we repeated the experiments of the preceding authors, at the same time examining the eggs in polarized light. We used the same hypertonic solution as Runnström and collaborators, i.e., 20 cc. of sea water and 6 cc. of a 2.5 M solution of NaCl. The eggs were put into the solution at intervals of 5–10 minutes and then observed under normal and polarized light.

The unfertilized egg treated by this solution shows a "polyhedric" plasmolysis (Fig. 2) and loses its cortical birefringence. Immediately after fertilization or parthenogenetic activation, a strong spherical plasmolysis is observed while a superficial hyaline layer becomes visible. It contains small granules and is of variable thickness in different specimens. The underlying cortical layer proper shows a vivid birefringence with polarization cross. Such a condition lasts 10–

15 minutes (experiments made in April, at $t° = 17° - 19°$) and therefore it coincides, in part at least, with the first birefringence cycle observed in normal eggs. After this first period, the angular plasmolysis appears, while in polarized light no cortical birefringence with polarization cross can be detected. The egg surface, especially the edges, appears luminous because of the presence of rather intensely birefringent granules. Such a luminosity always occurs in the cortical layer proper, while the superficial coat, less clearly defined than in the spherical plasmolysis, does not show any luminosity. The angular plasmolysis begins when the evolution of the sperm-aster is in progress.

Such a condition lasts until the metaphase or expanding monaster stage. Then, ca. 50–60 minutes after activation, the eggs in hypertonic solution show again spherical plasmolysis with intense cortical birefringence (Figs. 5, 7, 8). The controls in normal sea water undergo the second birefringence cycle. In this phase the superficial coat in the normal egg has reached its maximum thickness. In eggs treated with hypertonic solution the coat shows an undulated contour, as if, being inelastic, it could not follow the egg mass in its contraction (Fig. 6).

We did not observe a reappearance of the angular plasmolysis shortly before the end of the division, as described by Runnström.

In the parthenogenetic eggs, during the monaster regression the angular plasmolysis reappears, while in the controls birefringence disappears. In the second, third, etc., monaster cycle the spherical plasmolysis reappears, always in association with birefringence.

To summarize: these experiments show that the two types of plasmolysis mentioned by the authors quoted above and observed again by us, correspond fairly exactly with the cycles of cortical birefringence; namely, spherical plasmolysis corresponds with the phases of evident cortical birefringence, angular plasmolysis corresponds with the phases of lacking birefringence. We should like to point out again the fact, which will be discussed later, that during the first 10–15 minutes after activation, the cortical birefringence of the controls, at the conditions of temperature stated above, does not always manifest itself clearly. The hypertonic treatment, on the contrary, makes it very striking in all the eggs.

We also noticed that hypertony makes more evident a birefringence of the asters, already described by Schmidt (1939) and Monné (1945) (Figs. 5, 7, 8).

PLATE 1

All the figures are photographs of *Psammechinus miliaris* eggs at a magnification of 500 ×.

FIGURE 1. Unfertilized egg (polarized light): note the cortical birefringence.

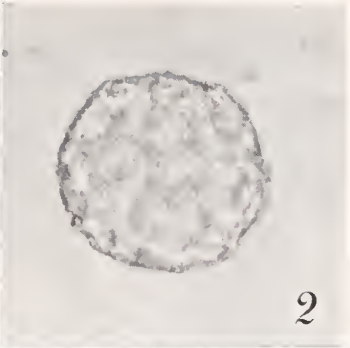
FIGURE 2. Unfertilized egg in hypertonic sea water (ordinary light): note the "polyhedric" plasmolysis.

FIGURE 3. An egg soon after fertilization (polarized light): note the birefringence of the fertilization membrane and the disappearance of the cortical birefringence.

FIGURE 4. An egg in the late metaphase stage (polarized light): the birefringence of the cortical layer is again evident.

FIGURES 5 AND 6. An egg in the late metaphase stage in hypertonic sea water as seen in polarized light (Fig. 5) and in ordinary light (Fig. 6). Note the smooth surface of the egg exhibiting a very brilliant birefringence (Fig. 5) and the wrinkled hyaline layer which does not exhibit any birefringence (Fig. 6). Note also the birefringence of the spindle as seen from a pole.

FIGURES 7 AND 8. Eggs in the beginning (Fig. 7) and late telophase (Fig. 8) in hypertonic sea water (polarized light): birefringence of the cortical layer and of the spindle-figure.



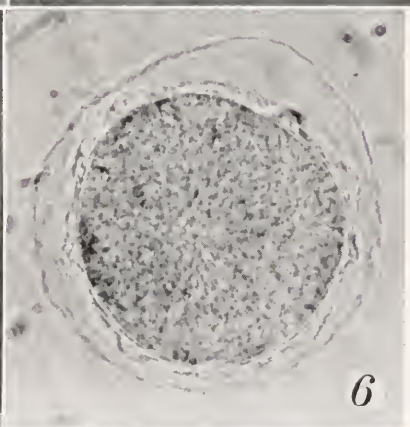


PLATE I

Colchicine experiments

The synchronism of the birefringence and the diaster and monaster cycles (in fertilized and parthenogenetic eggs respectively) as well as of the plasmolysis cycles leads one to think that all these phenomena are linked with one another. We were able to observe, however, that the first birefringence cycle (15–20 minutes after

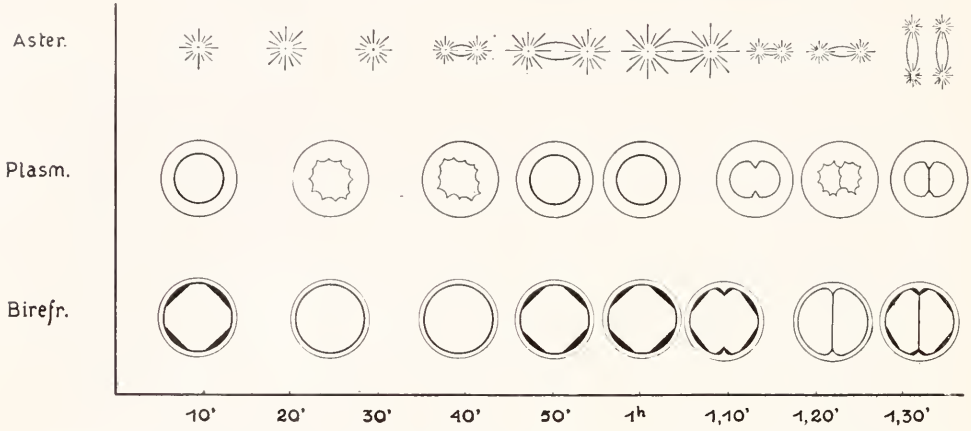


FIGURE 9. Diagram showing the time correspondence between the cycle of the cortical birefringence compared with the development of sperm-aster and spindle and the types of plasmolysis in fertilized eggs.

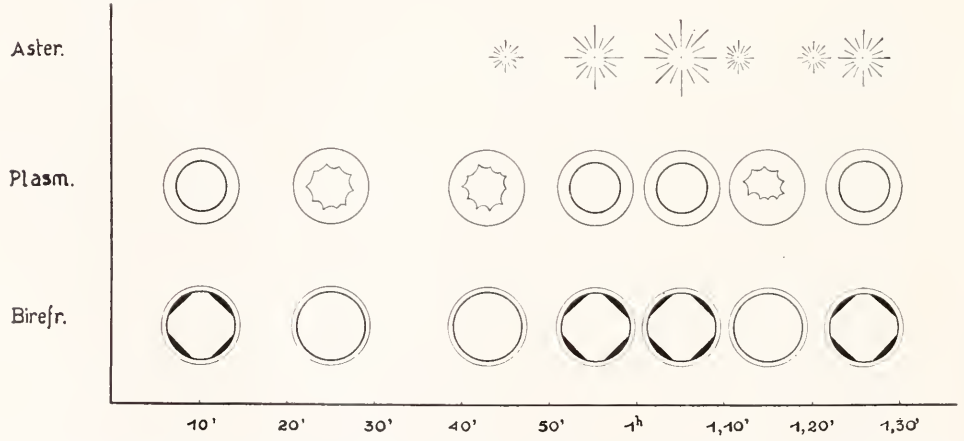


FIGURE 10. Diagram showing the time correspondence between the cycle of the cortical birefringence compared with the development of monaster and the types of plasmolysis in eggs activated with butyric acid.

fertilization) is not strictly correlated with the development of the sperm-aster, and in the parthenogenetic eggs, of course, is not connected with any phenomenon comparable to it. In order to test any possible correlation between the cortical birefringence and the aster cycles, we have treated the eggs with colchicine, with the aim of suppressing the aster.

The technique was the same as that used by Beams and Evans (1940), i.e., immersion of the fertilized or parthenogenetic eggs in a 0.0002 M solution of colchicine. In both series of experiments we observed the birefringence cycles, as already described, in complete absence of asters. The results are extremely clear cut, and therefore we can conclude that the cycles of the cortical birefringence, although normally synchronous with the cycles of the asters, are independent of them.

Furthermore the cycle of the two types of plasmolysis in colchicine-treated eggs proceeds exactly in the same way as in the controls. It is thus clear that the suppression of the aster by colchicine does not alter in any way the conditions of permeability of the cortical layer. Hence these experiments confirm Runnström's idea that the two types of plasmolysis are related to different conditions of the cortical layer only and not of the whole cytoplasm.

Conclusions and Discussion

From the above described investigations the following conclusions can be drawn:

1. The cortical layer of the unfertilized egg of *Psammechinus miliaris* shows a positive birefringence of yellow-orange color (Fig. 1).

2. Such birefringence disappears at fertilization or parthenogenetic activation (Fig. 3).

3. From 15 to 20 minutes after fertilization or activation, in certain conditions, a slight positive birefringence appears, of silver-white color. Hypertonic treatment makes it evident and constant also in cases in which in the controls it is faint or absent.

4. From 20 minutes to the end of metaphase, the cortical layer does not show any birefringence either in controls or in hypertonic treated eggs.

5. Beginning with the end of metaphase in fertilized eggs and with the starting of the monaster expansion in parthenogenetic eggs, a strong positive cortical birefringence appears, silver-white in color and very brilliant both in controls and in hypertonic treated eggs (Figs. 4, 5, 7, 8).

6. These cycles of variation of the submicroscopic structure of the cortical layer correspond exactly to the cycles of spherical and angular plasmolysis described by Runnström.

7. Aster inhibition by colchicine does not alter the cycle either of the cortical birefringence or of plasmolysis. The correspondence of the cycles of cortical birefringence, of sperm-aster and spindle in fertilized eggs, of monaster in parthenogenetic eggs and of the type of plasmolysis is summarized in the diagrams (Figs. 9, 10).

It is thus evident that after fertilization the cortical layer of the sea urchin egg undergoes cyclic variations of its submicroscopic structure, which are revealed by polarized light. Runnström, Monné and Broman (1943), on the contrary, did not find any variations of the cortical birefringence after fertilization, although they were surprised by the fact that corresponding to the modifications observed by Runnström (1923, 1928) in dark field, no analogous variations could be detected in polarized light.

We have already stated that the cortical birefringence which reappears soon after fertilization is very variable in intensity and constancy in the different lots and under different experimental conditions. Hypertonic treatment, however, makes it clear and constant in every egg. Furthermore we have noticed that in some eggs, a few minutes after fertilization (4–5 minutes) and, even more frequently, after parthenogenetic treatment, a very labile and short lasting birefringence appears, which soon fades away. All this indicates, to our opinion, that the cortical layer, after normal or parthenogenetic activation, up to ca. 15–20 minutes, is in a condition of particular lability in its submicroscopic structure, which probably has a physiological correspondence in the peculiar susceptibility to hypotonic solutions pointed out by Just (1922). Since the hypertonic treatment makes the birefringence evident, it can be assumed that, as an effect of activation, at first there is an increase of the distance between the molecules of the cortical layer, which however do not lose their orientation. Hypertony, by causing a decrease of volume, determines the approach of the molecules, thus showing their orientation.

We are not able, at the moment, to give an interpretation of the intimate mechanism of such morphological modifications, but we want to draw attention to the remarkable physico-chemical modifications which, as is well known, occur soon after fertilization. We regard it as probable that an important feature possibly concerned with the molecular orientation of the cortical layer, should be the variation of the distribution of some electrolytes and particularly of Ca, which have been observed by many authors soon after fertilization, e.g., Mazia (1937), Örström and Örström (1942), A. Monroy Oddo (in press). In fact, the importance of bivalent electrolytes (especially Ca) on the molecular orientation and attraction on complex ionic systems is known (Bungenberg de Jong et al., 1935; 1938). A loss of Ca may thus determine a decrease of the effective attraction between the molecules in the ionic layer, and thus a condition of greater hydration of the whole system. In this connection it is also interesting to note Öhman's (1945) observation that after fertilization a decrease of the free cephalin occurs, which, according to the author, is becoming linked to the proteins. That means a variation of the relations between the various constituents of the system, and it is not improbable that this fact also plays a rôle in determining a rearrangement of the composition and structure of the cortical layer.

Attention should also be called to a fact which might have some importance for the analysis of the action of butyric acid in the activation process. Unfertilized eggs put into butyric acid show a decrease in the intensity of the cortical birefringence, which at the same time becomes whitish. After a certain time in the acid the birefringence returns to the same condition as in unfertilized eggs. The period of decreased intensity corresponds approximately to the optimum time for a successful activation.

Following the first phase the second succeeds, lasting from 15–20 minutes up to 50–60 minutes after activation, and during this phase the birefringence disappears and angular plasmolysis sets in. One can interpret this fact by assuming that after the phase of molecular orientation characteristic of the unfertilized and freshly fertilized egg, a phase of disorientation follows. At this time the egg appears diffusely luminous in polarized light, and this is probably due to the lack of normality of the optic axis of the cortical layer.

As to the correspondence which we found between birefringence and spherical plasmolysis, and respectively isotropy and angular plasmolysis, we are inclined to think that in the first case the cortical layer, being formed by radially iso-oriented molecules, undergoes uniform contraction due to hypertony, while in the second case, because of the disordered molecular orientation, the resistance of the various points of the cortical layer varies, and thus it reacts to plasmolysis not by a uniform contraction, but by formation of wrinkles (cf., also, Runnström, Monné and Broman, 1943).

It might seem strange that the reaction of the fertilized egg to hypertony, while its cortical layer has an orientated structure, is different from the reaction of the unfertilized egg, where the cortical layer also has an orientated structure apparently entirely similar. The unfertilized egg in fact, as we said, in hypertonic solution contracts with a "polyhedric" surface and loses its cortical birefringence. We have already mentioned the chemical and structural variations which probably occur in the cortical layer of the egg after fertilization and in particular the loss of Ca and perhaps also of some of its lipidic components (cephalin ?). This results in a condition of greater softness than in the unfertilized egg. In the latter, the Ca content of the whole egg and probably also that of the cortical layer is greater, and this causes a greater condensation of its molecules. This is probably the cause of a higher rigidity upon which the characteristic type of plasmolysis depends. It is not improbable, however, that in this phenomenon a rôle is played also by particular relations of the molecules of the cortical layer with the subcortical proteins, as the researches of Runnström, Monné and Broman (1943) seem to suggest. They found that trypsin-treated unfertilized eggs in hypertonic solution contract with a smooth surface, without losing their cortical birefringence.

Apparently this mechanical interpretation fits the observed facts better than Öhman's (1945) explanation. He considers the cortical layer as a lipo-proteic film, in which the quantity of proteins and lipids may vary. Of course our interpretation does not exclude the possibility that during the two phases, viz., orientation and disorientation, the relations between cortical lipids and subcortical proteins may also vary. Herlant's (1920) observations on the variations of susceptibility of the egg to fat solvents which occur between fertilization and first cleavage do not seem to coincide well with the rhythms of plasmolysis; they seem rather to indicate the existence of phases of greater and lesser susceptibility which are not easy to interpret because of their irregularity. At any rate, it would probably be worthwhile to repeat such experiments in connection with observations in polarized light, taking into account especially Bungenberg de Jong's researches on the mode of action of organic and inorganic compounds on complex ionic systems, and in particular on the influence they have on the length of the carbon chain.

It is perhaps pertinent to recall here an observation by Öhman (1945), viz., that the formation of bubbles by the action of heat is entirely inhibited soon after fertilization i.e., during the contraction phase. Later on the frequency with which eggs show bubbles gradually increases and reaches a maximum between 20 and 30 minutes after fertilization. Then another phase sets in, characterized by a lesser frequency of bubble formation (not total inhibition as soon after fertilization) which extends up to the anaphase of the first mitosis, at which time it increases again. The coincidence of this rhythm with the phases of cortical orientation and disorienta-

tion which we have described is very striking. The fact might possibly mean that the bubble formation is greatly facilitated by the molecular orientation of the cortical layer, as was to be expected on the ground of Bungenberg de Jong and Bonner's (1935) researches on bubble formation in drops of coacervates.

In conclusion we believe that the lipidic character of the cortical layer of the egg is not lost at fertilization, although rearrangements in its chemical composition and consequent variations of physical properties may well take place. Furthermore we recall that one of us (Monroy, 1945) has already pointed out that the cortical birefringence which reappears after fertilization, during the second cycle, has the same characters and the same quantitative value as in the unfertilized egg.

Furthermore the following fact found by us seems interesting: while birefringence conditions and type of plasmolysis are undoubtedly linked to one another, no linkage seems to exist between these phenomena and the aster cycles, although some synchronism is undeniable. The colchicine experiments allowed us to demonstrate that the birefringence-permeability cycle can proceed entirely undisturbed when asters are inhibited. It is also a remarkable fact that in parthenogenetically activated eggs, at the end of the monaster cycle, angular plasmolysis reappears as in fertilized eggs at the end of cleavage. Clearly enough we are dealing here with a rhythm characteristic of the activated egg independent of cellular division.

Our researches have not thrown light on the submicroscopic structure of the superficial hyaline layer, which, as is well known, appears after fertilization and is more evident in hypertony. It did not show any birefringence in our experimental conditions. We were able to observe that the cortical birefringent layer is always underlying the hyaline coat (as already stated by Runnström, Monné and Broman, 1943) and that the latter, especially during the second phase of spherical plasmolysis, is apparently non-elastic, and therefore, being unable to follow the egg in its contraction, shows wrinkles and undulations. This observation seems to be in favour of the presence of some sort of cleavage between the hyaline coat and the cortical layer, and that would explain how E. B. Harvey (1934) was able to shift it entirely from the egg by means of centrifugation.

Summary

The authors have investigated the structural variations of the cortical layer of *Psammechinus miliaris* egg after fertilization or parthenogenetic activation, by means of polarized light, hypertonic treatment and colchicine.

They found regular cyclic variations of the birefringence of the cortical layer. A first inconstant cycle of birefringence appears at 15–20 minutes after fertilization or parthenogenetic activation. A second, more intense and constant cycle appears at the end of metaphase up to the telophase of the first cleavage, or, in parthenogenetic eggs, at the expansion of the monaster.

In eggs treated with hypertonic solution, "spherical plasmolysis" corresponds to the birefringent phases, while "angular plasmolysis" corresponds to the non-birefringent phases.

Colchicine, which inhibits the aster formation, does not alter either the birefringence or the plasmolysis cycles and their synchronism. The latter two phenomena are thus linked to one another, but independent of the cycles of the aster.

The meaning of the facts and their relations to the physico-chemical phenomena occurring during activation are discussed.

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PHYSIOLOGICAL OBSERVATIONS UPON A LARVAL EUSTRONGYLIDES. XI. INFLUENCE OF OXYGEN TENSION ON THE AEROBIC AND POST-ANAEROBIC OXYGEN CONSUMPTION.

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Harnisch (1932a, b; 1933; 1935a, b, c) has emphasized that the respiration of parasitic worms is dependent on the oxygen tension at least up to a tension of 760 mm. Hg. He has also shown that the post-anaerobic oxygen consumption, the repayment of an oxygen debt, shows the same relationship in a number of free living organisms, the normal aerobic respiration of which is much more independent on the tension. He has used this argument as one of the cornerstones for his hypothesis that the respiration of parasitic worms and the post-anaerobic oxygen consumption of free living animals is governed by another set of enzymes (secondary aerobiosis) than the normal aerobic respiration (primary aerobiosis) of the latter. Von Buddenbrock (1939) has questioned this view in so far as free living animals are concerned, pointing out that the tension at which the oxygen consumption begins to decline is not static but changes with the intensity of the oxygen consumption.

Since Harnisch used for his investigations on parasites only intestinal and bile-duct helminths, it appeared worthwhile to study in this respect the larvae of *Eustrongylides ignotus* which definitely lead in nature a much more aerobic life than the former (von Brand, 1942). It was hoped to decide between Harnisch' and von Buddenbrock's views by using larvae at various levels of metabolism, the changes being partly induced by the stimulating effect of the potassium ion (von Brand, 1943), partly by various temperatures. Although this hope was not completely fulfilled, the experiments in themselves are of sufficient interest to warrant publication.

Material and Methods

In the respiration experiments reported upon previously in this series (von Brand, 1942, 1943) *Eustrongylides* larvae freshly isolated from the cysts which they normally occupy in *Fundulus* were used almost exclusively. It was then found that they showed initially a trace of excess oxygen consumption. This phase had to be eliminated for the present experiments. The worms were therefore isolated in batches from 3 to 5 specimens, weighed, and kept over night in a shallow layer of either 0.85 per cent NaCl or 1.14 per cent KCl, that is, under conditions allowing for a completely aerobic metabolism. After this preliminary period their oxygen consumption was studied at various oxygen tensions with a minimum of 1½ hour for each tension. The fluid in the respiration vessels was the same as that used during

[1] The author is indebted to the Elizabeth Thompson Science Fund for a grant towards the purchase of the respiration apparatus used in this investigation.

the preliminary period. The oxygen determinations were carried out by means of a Warburg apparatus. The gas mixtures were passed for 20 minutes through the vessels before closing the manometers; they contained oxygen at the following tensions: 760, 160, 34, and 6 mm. Hg. Usually from 2 to 3 different tensions were used consecutively with one batch of worms. Care was taken to employ the highest tension first in order to avoid an excessively high oxygen consumption if the worms should acquire an oxygen debt at the lower tensions. Previous experiments had shown that the respiration of the larvae remains virtually constant over long periods if the conditions are not changed. Any decline in oxygen consumption following the replacement of a gas mixture with high oxygen tension by one containing less oxygen could consequently safely be ascribed to the lowered tension. The experiments of these series were carried out at 40.2, 28.5, and 18.8° C.

A slightly different procedure had to be employed in the series in which the dependency of the post-anaerobic oxygen consumption on the tension was studied. The isolated and weighed worms were first exposed, in 0.85 per cent NaCl, for a period of 16 hours to anaerobic conditions, using the method previously described (von Brand, 1942). In order to get as uniform an oxygen debt as possible, the worms were subjected to this anoxic period at 37° C. regardless of the temperature at which the post-anaerobic oxygen consumption was studied subsequently. The repayment of an oxygen debt is a transitory phenomenon; after an initial high rate, the oxygen consumption declines slowly until it reaches finally the pre-anaerobic level. The phase of high oxygen consumption will last longer at low than at high temperatures if an equal oxygen debt is repayed at both temperatures. These facts precluded obviously the consecutive use of various gas mixtures with one and the same group of animals. Instead, a new batch of worms was used for each experiment at each tension and only the oxygen consumed during the first half hour was taken into consideration. These experiments were carried out at the same tensions and temperatures as those mentioned above; an additional series was performed at 9.8° C.

Results and Discussion

A summary of the experiments is presented in Table I. It is evident that the dependency of the oxygen consumption on the tension is much more pronounced at high than at low temperatures if the comparison is made between series performed under otherwise equal conditions. In the aerobic NaCl series, for example, the oxygen consumption was dependent, at 40.2° C., on the tension over the entire range of tensions studied, while at 18.8° C., it was almost constant in the range of 760 to 34 mm. Hg.

The post-anaerobic oxygen consumption was, at each temperature studied, about three times as high as the normal aerobic respiration in NaCl solution when the values found at an oxygen tension of 760 mm. Hg are compared.

If, as von Buddenbrock (1939) maintains, the intensity of the respiratory rate decides at what tension the consumption begins to decline, one should expect that at each temperature the curve for the post-anaerobic oxygen consumption should be steeper than that of either the aerobic NaCl or KCl series. The observations confirm, on the whole, this view, especially at tensions below 160 mm. Hg. Between 760 and 160 mm. Hg there was hardly a significant difference between the three

TABLE I

Influence of oxygen tension on the normal aerobic and the post-anaerobic oxygen consumption of larval Eustrongylides ignotus. Under oxygen consumption the mean values with the standard error of the means and, in brackets, the extremes are listed

Oxygen tension, mm. Hg	Temp. °C.	Aerobic, 0.85 per cent NaCl				Aerobic, 1.14 per cent KCl				Post-anaerobic, 0.85 per cent NaCl			
		760	160	34	6	760	160	34	6	760	160	34	6
Experiments, No.	40.2	14	19	12	8	7	15	8	12	8	8	8	8
O_2 consumption mm.³/g./half hour		139±11 (75, 216)	104±5 (69, 140)	87±4 (62, 110)	27±2.2 (19, 36)	174±9 (137, 214)	148±9 (100, 214)	100±9 (62, 149)	22±2.8 (11, 37)	373±7 (349, 412)	318±25 (246, 468)	131±15 (68, 204)	38±4.8 (16, 58)
Experiments, No.	28.5	16	24	14	20	15	16	15	15	12	12	12	10
O_2 consumption mm.³/g./half hour		67±3.3 (43, 88)	51±3.2 (31, 84)	48±3.9 (30, 73)	32±2.5 (10, 52)	86±3.7 (63, 107)	69±4.5 (39, 117)	70±3.0 (53, 89)	27±3.0 (10, 51)	196±5 (164, 224)	177±6 (161, 215)	91±7.7 (42, 132)	30±4.4 (9, 47)
Experiments, No.	18.8	12	12	12	12	12	12	12	12	11	12	12	11
O_2 consumption mm.³/g./half hour		30±1.9 (16, 39)	29±2.3 (19, 42)	28±1.7 (19, 40)	13±0.8 (8, 20)	41±2.5 (25, 54)	38±1.7 (29, 48)	37±2.9 (22, 54)	13±2.1 (5, 28)	87±2.9 (76, 101)	83±3.6 (71, 97)	61±2.3 (48, 72)	12±2.0 (5, 23)
Experiments, No.	9.8									10	12	12	12
O_2 consumption mm.³/g./half hour										46±1.9 (38, 57)	43±1.9 (33, 52)	39±1.5 (27, 48)	11±1.2 (4, 18)

conditions studied, with the exception of the 40.2° series which showed the postulated relationship. To make von Buddenbrock's view of the essential similarity between normal and post-anaerobic oxygen consumption entirely acceptable, another prerequisite would have to be met. One should expect that the dependency of the post-anaerobic oxygen consumption on the tension should be the same as the aerobic one if one compares series in which the intensity was equal at a tension of 760 mm. Hg, i.e., if one takes as basis of comparison post-anaerobic experiments performed at lower temperatures than the aerobic ones. The post-anaerobic experiments performed at 18.8° C. showed at 760 mm. Hg exactly the same intensity in oxygen consumption as the 28.5° C. KCl series. Similarly, the rate of oxygen consumption in the 9.8° C. post-anaerobic series was quite similar to that found in the 18.8° C. KCl series. In both cases as slightly greater dependency on the tension was observed in the post-anaerobic series; the differences are, however, not statistically significant.

While the present experiments seem, therefore, to support von Buddenbrock's view, they do not necessarily as yet completely disprove Harnisch's conception of "primary" and "secondary aerobiosis." Harnisch has found evidence that the respiration of parasitic worms is almost exclusively of the second type, that is, it corresponds to the post-anaerobic oxygen consumption of free living animals. One would then also expect identical curves in cases where the metabolic levels coincide. Although the larvae of *Eustrongylides ignotus* resemble in their general metabolism unquestionably free living animals more than they do intestinal helminths, it cannot be stated categorically that they do so in the question under consideration. To decide definitely between Harnisch and von Buddenbrock, experiments similar to those described in the present paper should be performed on free living animals. Certainly, von Buddenbrock's criticism of Harnisch's failure to consider the intensity factor is justified, but that is hardly sufficient to reject the latter's views without further experimentation.

One further point deserves brief discussion. Von Buddenbrock (1939) has pointed out that the reasons for assuming a dependency of the oxygen consumption on the tension are occasionally rather insecure because the gases going into purely physical solution are neglected. He cites the example of the sea urchin *Sphaerechinus granularis* which shows for a certain period an increased oxygen consumption if it is transferred from sea water with normal oxygen content into water containing an abnormally high oxygen content. Analyses of the coelomic fluid revealed that the greatest part of this excess oxygen had simply gone into physical solution and had not participated in the metabolic processes. Such an explanation cannot be applied to the *Eustrongylides* larvae. Some of the worms were, after their metabolic level had been ascertained at an oxygen tension of 160 mm. Hg, kept up to 6 hours in pure oxygen at 40.2° C. They showed during the entire time a regular oxygen consumption which was all the time higher than that found in the preliminary period. Obviously, we are dealing here with a case of true dependency on the tension, just as it occurs in actinians which are, as is known since Henze's (1909) investigation, a classical example for this type of relationship.

It should finally be noted that the exposure to pure oxygen for 6 hours did not seem to harm the *Eustrongylides* larvae. They differ in this respect from the intestinal helminth *Ascaris* which is rapidly killed by oxygen of high tension (Laser,

1944). This difference may have a biological basis in the fact that the latter worm lives normally in a much oxygen poorer habitat than the former.

Summary

1. The oxygen consumption of larval *Eustrongylides ignotus* shows a greater dependency on the oxygen tension at high than at low temperature.

2. The post-anaerobic oxygen consumption is more dependent on the tension than the respiration of larvae that were exposed previously to well oxygenated surroundings, if experiments performed at equal temperatures are compared. This difference disappears almost completely, however, if experiments are compared in which the intensity of the oxygen consumption was equal in both sets at a tension of 760 mm. Hg.

3. The implication of these data is discussed on the controversy between Harnisch and von Buddenbrock as to whether it is justified to distinguish between "primary" and "secondary aerobiosis."

4. The dependency of the oxygen consumption on the tension is a true one and is not only simulated by oxygen going into physical solution in the body fluids at higher tensions.

5. The larvae of *Eustrongylides,* in contrast to *Ascaris,* are not killed by a 6-hour exposure to pure oxygen.

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RELATIONS BETWEEN METABOLISM AND MORPHOGENESIS DURING REGENERATION IN TUBIFEX TUBIFEX I

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There have been two prime viewpoints regarding the relation of metabolism to morphogenesis. One advanced by Child and his students has considered rate of metabolism in relation to the organismal factors initiating and directing the course of morphogenesis. Barth (1938, 1940a, 1940b), supporting Child's view, has suggested that increased oxygen tension in the tissues adjacent to a wound may furnish a primary stimulus for regeneration in hydroids; but he found that rate of regeneration was much more markedly stimulated by high oxygen tension than was rate of oxygen consumption. Thus the effect on regeneration may not have been mediated through an effect on total aerobic metabolism. The other viewpoint, advanced by Tangl (1909) and more recently by Tyler (1933, 1936, 1942), has considered rate of metabolism in relation to the release and utilization of energy in morphogenetic processes, particularly differentiation. Tyler concluded from his work on sea urchin embryos that differentiation requires the expenditure of metabolically released energy in addition to that required for maintenance, and that this energy does not become resident in structure but is released as heat during morphogenesis. This view is consistent with the work on "activity metabolism" (cf., Fisher et al., 1942, 1944).

The present study was undertaken to analyze further the relations between metabolism and morphogenesis from both viewpoints. In the regenerating annelid, measurements of morphogenesis may be made by counting the number of new segments produced (Stone, 1932 and Coldwater, 1933). The formation of a new segment is not a simple event, but involves distinct stages. This allows estimation of the rates at which various processes in regeneration proceed. Metabolism may be separated into fractions by means of poisons, and the relation of the activity of particular cellular respiratory systems to morphogenesis may then be tested. Two modes of approach were used in this study: oxygen consumption and respiratory sensitivity to poisons were measured at various times during regeneration (results reported in this paper), and the effects of continuous poisoning and of high and low oxygen tension were determined by measuring the progress of regeneration (results to be reported later).

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Materials and Methods

Worms of the species *Tubifex tubifex*[1] were rigorously selected for uniform size and condition (3.5 to 5.0 centimeters in length, a light, smoothly graded color, no signs of previous regeneration, no signs of breeding condition). For a week before use and between determinations the worms were kept in frequently changed tapwater at about 17°C. A 0.2 per cent solution of chloretone was used as an anaesthetic during amputation of the posterior two-fifths of each worm and during examination under the microscope. Individual worms were kept in anaesthesia no longer than ten minutes every third day (cf., Stone, 1932). Stage and rate of regeneration were estimated at intervals of several days. Three stages in the formation of a new segment were clearly distinguishable *in vivo*. First the new segment appeared as a narrow shadow perpendicular to the longitudinal axis of the worm. Later the new segment was slightly longer and the septum appeared as a pair of fine sharp lines. Finally the new setae were visible as small refractile bodies. The three stages were considered to be stages of localization, early differentiation, and later differentiation respectively (cf., Stone's description of the cytology and histology of regeneration in *Tubifex tubifex*). The number of segments in each of the stages was recorded separately. After regeneration had proceeded for a week or more, all stages of segments were present in the new tail, but segments which were hard to classify were few in number. "Rate of localization"[2] was calculated as the increase in total number of segments per worm per day; "rate of early differentiation" as the increase in number of segments in all stages of differentiation, and "rate of later differentiation" as the increase in number of segments with setae.

Most of the determinations of oxygen consumption were made by the Warburg manometric method, at 25°C. Worms for these determinations were taken in groups of twenty-five to forty, collected into a ball, rolled on filter paper to free them of excess water, placed in a pan of platinum foil and weighed quickly on a torsion balance. Consecutive weighings were found to deviate by less than one per cent. A difficulty in use of intact animals in the Warburg apparatus was encountered in the differences in degree of dispersion of the worms over the bottom of each flask. Probably the marked ability of *T. tubifex* to contract "oxygen debt" (Harnisch, 1935) was brought into play when the worms remained in a ball (average oxygen consumption in five determinations during which the worms remained in a ball was 0.12 milliliter per gram wet weight per hour compared to 0.16 milliliter for dispersed worms). Fortunately the worms spread out uniformly in most cases and only those determinations throughout which the worms were uniformly dispersed were included when calculating averages.

Successive determinations were made on the same worms at intervals of several days and the progress of regeneration was also measured. That this routine was not unduly injurious to the worms was indicated by a normal rate of regeneration and a casualty rate only slightly higher than that of worms upon which no manometric determinations were made; also the oxygen consumption of intact worms used as control remained constant within the limits of error throughout each series of

[1] Identification confirmed by Dr. R. G. Stone, University of Kansas City.

[2] This is the same as rate of regeneration as used by Stone (1932) and Coldwater (1933), although these authors did not calculate rate on a daily basis. From their published data on comparable worms the curve in Graph 1 may be duplicated.

determinations. These intact worms were anaesthetized and examined as were the experimental animals, injured individuals being removed from the group. About 10 per cent of the worms in each group had been removed by the end of the experiments.

A modification of the method reported by Howland and Bernstein (1931) was used for measuring oxygen consumption by individual worms. Each worm was drawn into a capillary tube 0.5 millimeters in diameter. By shifting the worm in a drop of water back and forth in the tube, air was drawn in at each end, then a drop of 5 per cent potassium hydroxide solution. Finally one end was sealed with paraffin oil and the other with vaseline (see Fig. 1). The tube was mounted in a

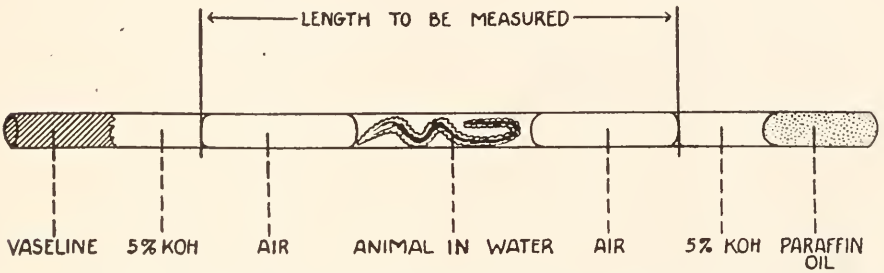


FIGURE. 1. Diagram of respirometer used in modified Howland-Bernstein method of measuring oxygen consumption (much enlarged).

vertical position on a glass plate in a glass-walled constant-temperature bath at 21°C. About fifteen such tubes were handled at a time. The distance between upper and lower air-KOH interfaces was measured using a horizontal microscope with a calibrated adjustment. The inside diameter of the capillary was measured exactly and the volume of oxygen consumed was calculated directly. A wormless tube served as a thermobarometer. The movement of the worm in the tube was found to furnish an efficient stirring mechanism in the fluid part of the system, and it was calculated that the capillary was not fine enough to hinder diffusion in the gaseous phase. After a series of readings, the capillary tubes were broken at the air spaces and the worms removed uninjured. Measurement of each worm was then carried out by anaesthetizing it and taking the diameter at four or five points and the length. The volume of the worm was computed as a series of cylinders.

DATA ON WORMS NOT UNDERGOING REGENERATION

As determined both by use of the Warburg apparatus and by the method just described, the rate of oxygen consumption of normal worms was within the range of rates reported by other workers using various methods (Dausend, 1931; Harnisch, 1935; Brazda and Rice, 1940). In agreement with the last named investigators, it was found that size of worms from 2.5 to 5.0 centimeters in length made no significant difference in rate of oxygen consumption. Concentrations of potassium cyanide from 2×10^{-3} M to 2×10^{-6} M had no inhibitory effect on oxygen consumption by normal worms (see Table I). Cyanide in a concentration of 2×10^{-2} M was rapidly lethal, but nevertheless did not decrease oxygen consump-

tion as drastically as its toxicity might suggest. Comparison with the data reviewed by Commoner (1940) shows that the total oxygen consumption by *T. tubifex* is of the same absolute magnitude as the cyanide-stable fraction of respiration in many other animals and tissues for which such data are available.

TABLE I

Oxygen consumption by intact worms in various concentrations of potassium cyanide

Solution	Q_{O_2}	Number of determinations
Tapwater	0.16	22
2×10^{-6} M KCN	0.16	2
2×10^{-5} M KCN	0.16	6
2×10^{-4} M KCN (non-lethal)	0.15	5
2×10^{-3} M KCN (semi-lethal)	0.19	2
2×10^{-2} M KCN (lethal)	0.11	1

Note: Determinations were made using standard manometric procedure at 25°C.

Respiratory quotient was found to average 0.68 for normal starving worms. The data agree with those of Brazda and Rice and suggest non-carbohydrate metabolism, possibly protein or fat metabolism. Such a type of metabolism is also expected from the cyanide-stable character of the respiration (cf., Commoner's review) as well as from the starving condition of the worms.

EXPERIMENTAL RESULTS

In preliminary work measuring the oxygen consumption of individual worms (see Table II) it was found that during the first few days of regeneration oxygen

TABLE II

Oxygen consumption by individual worms

Condition	Oxygen in mm.³ per mm.³ of worm per hour					
	0 days	1 day	8 days	11 days	15 days	19 days
Normal intact	0.10	0.10	0.11	0.09	0.09	0.09
Normal cut (regenerating)	0.10	0.12	0.14	0.17	0.15	0.15
X-rayed intact	0.08	0.09	0.07	0.09	0.08	0.11
X-rayed cut (inhibited)	0.10	0.11	0.09	0.11	0.10	0.11

Note: Determinations were made using capillary tubes at 21°C. Probable error was ±0.01.

consumption proceeds at a rate only slightly higher than normal. Later a marked increase in rate of oxygen consumption appeared: 0.08 mm.³ per mm.³ of worm per hour, or 85 per cent above normal. Subsequently the rate of oxygen consumption decreased. It was found that worms in which regeneration had been inhibited

by X-ray treatment showed no similar increase in oxygen consumption. It was considered that the increased oxygen consumption was associated with some process occurring during regeneration. In this preliminary work no counts of segments had been made, but the data of Stone (1932) were used for comparison. It was suggested that differentiation might be the process with which the increased oxygen consumption was associated. It was thought possible that the increase in oxygen consumption might be cyanide-sensitive as in the case of grasshopper embryos (Bodine et al., 1934, 1940). To test this possibility as well as to check results thoroughly using a standard method, experiments were set up in which the Q_{O_2} of regenerating worms in tapwater and in dilute cyanide solution (2×10^{-4} M KCN) was determined using Warburg manometers. Detailed observations of the progress of regeneration were made on the same worms. A total of nine groups of worms, 303 individuals, were followed through regeneration in three series of experiments. Four groups of worms, 137 individuals, served as control. Results of the manometric determinations are summarized in Table III, each figure being the average of

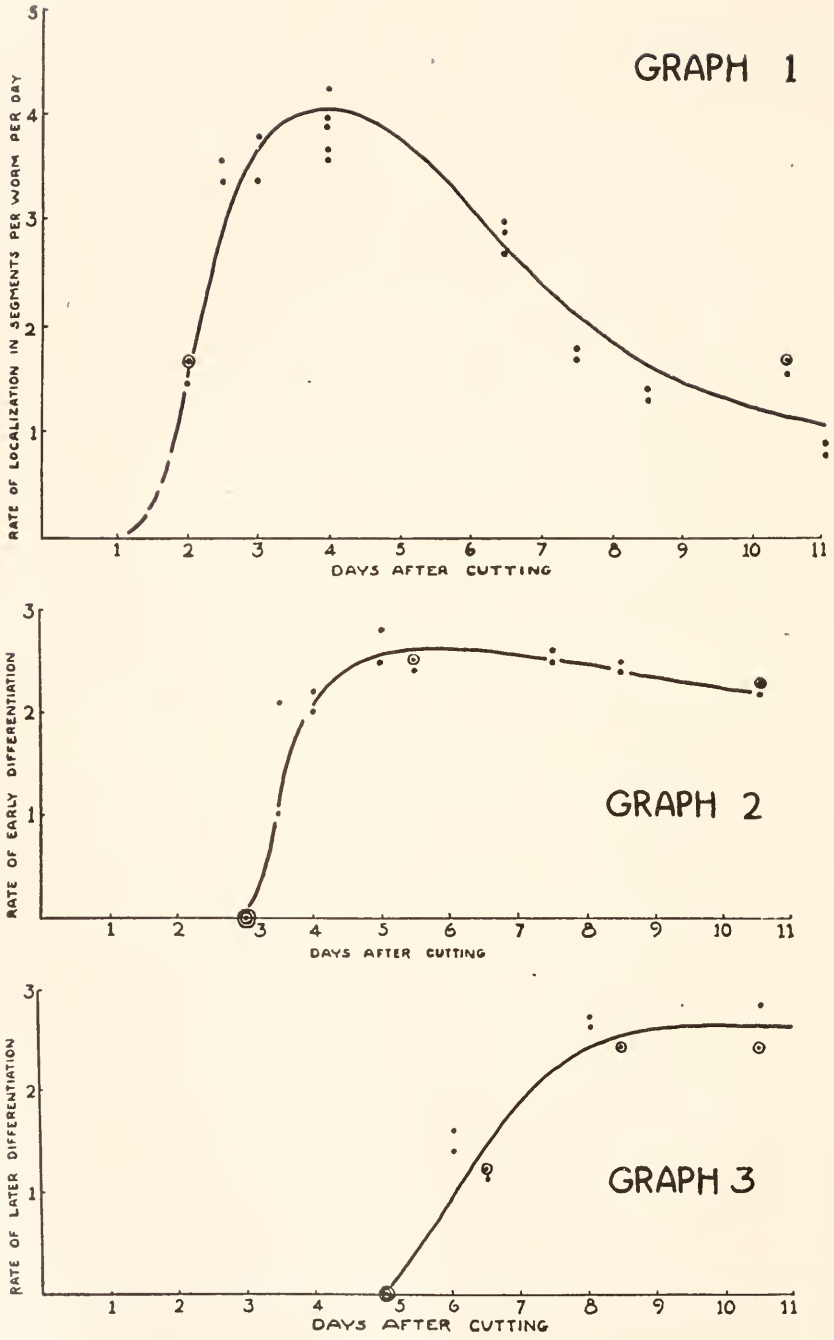
TABLE III

Oxygen consumption by groups of worms

Days	Predominating regenerative processes	Regenerating worms		Concurrent control	
		Q_{O_2}	$Q_{O_2}^{KCN}$	Q_{O_2}	$Q_{O_2}^{KCN}$
2	Mobilization of neoblasts and beginning localization	0.18	0.20	0.17	0.17
3	Localization	0.17	0.16	0.16	0.16
4	Localization	0.16	0.15	0.16	0.15
5	Localization and early differentiation	0.17	0.16	0.16	0.15
6	Early differentiation	0.16	0.15	0.17	0.17
8	Early differentiation	0.18	0.17	0.16	0.15
12	Later differentiation	0.24	0.24	0.16	0.15
13	Later differentiation	0.24	0.25	0.15	0.15

Note: The error when calculating from data on thirty-minute intervals was ±0.009. When calculating from data on the whole run, the error was reduced to about ±0.003. Mean deviation of separate averages from the grand averages listed in this table was ±0.008.

determinations on from two to five groups of worms. Data on progress of regeneration are summarized in Graphs 1, 2, and 3. After two days of regeneration, during which localization occurred in the first new segment from the neoblasts in the blastema, oxygen consumption was slightly above normal. Between three and five days, the period of maximal rate of localization, oxygen consumption was only slightly if at all above normal. While early differentiation was proceeding at its maximum rate six days after removal of the tail, oxygen consumption was strictly normal. But while later differentiation was proceeding, rate of oxygen consumption was increased: at twelve days the rate was 0.08 milliliters per gram per hour higher than the control, i.e., 50 per cent higher. At this time rate of later differentiation was at a maximum. The determinations using the Warburg method confirm in



Note: Mean deviation within each group of worms (twenty-five to forty individuals) ranged from 0.2 to 0.4 segments per worm per day, increasing with time.

general the results of the earlier determinations using individual worms in capillary tubes.[3] At no time was a significant cyanide-sensitive fraction of respiration found.

A summary of data on loss of weight by the worms involved in this study is given in Table IV. These data indicate not only that the regenerating worms lost

TABLE IV

Original length of worms in centimeters	Condition	Average weight per worm at first weighing after removal of tail (2, 3, 4 days)	Average weight per worm at last weighing (12, 13 days)	Average percentage loss per day
3.5 to 4.0	Regenerating	1.36 mg.	0.94 mg.	3.09
	Normal	1.92 mg.	1.57 mg.	1.82
4.0 to 5.0	Regenerating	2.07 mg.	1.57 mg.	3.01
	Normal	2.53 mg.	2.22 mg.	1.51

almost twice as much weight as the control, but also that the initial differences in weight between experimental and control groups due to removal of the tails cannot account for either the increased oxygen consumption or the increased loss of weight. The latter point is clear from a comparison between the larger regenerating worms (4.0 to 5.0 centimeters original length) and the smaller normal worms (3.5 to 4.0 centimeters in length).

DISCUSSION

Morphogenesis, the origin of form, involves the production of the specific structural chemicals of protoplasm, their orientation into submicroscopic structure, and the summation of submicroscopic structural differences into microscopic and macroscopic structure. Morphogenesis depends upon metabolism in that anabolic processes produce the particular compounds which become oriented into structural parts and catabolic processes must release in usable form such energy as may be required, if any, in morphogenetic processes. Morphogenesis may involve work in the thermodynamic sense according to either of two conditions: (1) there may be free energy of formation of the structure such that the energy becomes resident in chemical and physical structure, and/or (2) the synthesis of materials, their transport to particular positions, and their fixation in these positions, may require energy in such a way that the energy is ultimately dissipated as heat, even as the energy required to move an object horizontally from one point to another is ultimately dissipated as heat.

Tangl (1909), Farkas (1903) and Bohr and Hasselbalch (1903) defined "Entwichlungsarbeit," "work of development," as the total energy dissipated during embryonic development. In a number of different animals the Entwicklungsarbeit was found to be equal to about one-third of the total energy content of the unincubated egg. Needham (1931) criticized the concepts of Tangl and contended that

[3] The differences in general level of oxygen consumption are probably due to the difference in temperature at which determinations were made, i.e., Warburg determinations at 25° C., and capillary tube determinations at 21° C. (Brazda and Rice, 1940, found that oxygen consumption was increased about 40 per cent when the temperature was raised from 25° C. to 30° C.) and to inequality of unit volume to unit weight.

"work of development" should be a potential energy resident in structure, i.e., should be "work of differentiation." He further pointed out in reviewing the work of Bohr and Hasselbalch, that the energy resident in mechanical structure must be either non-existent or extremely small in amount. Essentially, the difficulties arose from the inadequacy of controls: no measure of an energetic cost of maintenance separate from an energetic cost of development was available in the work of Tangl, Farkas, and Bohr and Hasselbalch.

Tyler (1933) found that normally developing dwarf sea urchin embryos consumed more oxygen than normal-sized embryos in reaching the same stages of development. He interpreted his results in terms of a metabolic cost of differentiation. Tyler's concept, different from that discussed by Needham, involves the expenditure of energy in morphogenesis but not necessarily the storage of energy in visible structure. His investigations of giant embryos gave evidence consistent with his interpretation and he tried further to dissociate differentiation, growth, and maintenance in his material, but was unsuccessful. He stated (1936), "It has been concluded from earlier work that energy is required for the processes of embryonic differentiation, although the quantities involved could not be estimated."

Some of the difficulties encountered in studies of morphogenesis in embryonic development are avoided when morphogenesis is investigated in regeneration. Intact animals may be used in measuring the metabolism associated with maintenance,[4] and comparison between the metabolic rates of regenerating and of intact animals gives a measure of metabolism associated with morphogenesis. Increased rate of oxygen consumption during regeneration has been reported in Planarians (Coldwater, 1930). The additional metabolism appeared immediately after cutting and may have been associated with proliferation and increased numbers of the formative cells in this case. Coldwater chose this interpretation. However, it has not been demonstrated that either formative cells or other cells of an embryonic nature have intrinsically high respiratory rate. Tumor cells, for instance, have been found to have a relatively low Q_{O_2} (Warburg, 1930). Since these may be considered embryonic cells in which determination and differentiation are not proceeding, their metabolism may be considered entirely associated with maintenance and proliferation. On this basis, maintenance of embryonic cells should not, in itself, lead to increased respiration. From an examination of Needham's comprehensive review of the investigations of metabolism of embryos it is clear that the magnitude of rate of oxygen consumption is not greater than that of certain adult tissues. It is possible that in regenerating Planarians the additional metabolism was associated with truly morphogenetic processes rather than with proliferation and maintenance, but the data do not allow a distinction between the possibilities.

In the present work a distinction is possible. During regeneration in *T. tubifex* the rate of oxygen consumption was found to remain normal for the first week. During the second week the oxygen consumption was found to increase to well above normal. Because the markedly increased oxygen consumption did not appear during early stages of regeneration, it certainly was not associated with mobilization or proliferation of the neoblasts. That it was not associated with increase in size

[4] In the present work the data provide an empirical check on this assumption: since the Q_{O_2} remained normal during most of the first week of regeneration, the aerobic metabolic cost of maintenance of regenerating worms must be the same as that of intact worms.

of the new segments is clear from the consideration that increase in rate of oxygen consumption appeared before marked increase in the size of the new tail; during the first two weeks the regenerant tail constituted only a very small fraction of the total volume of the worm (less than one-twentieth). Hence the additional oxygen consumption may be considered to be associated with morphogenesis proper. From the present data it is not possible to distinguish whether the additional oxygen consumption occurred in the regenerant tail alone or whether the older tissues also consumed oxygen more rapidly. If the increase be referred to the regenerant tail alone, the Q_{O_2} of this part must have been extremely high. However, in estimating a metabolic cost of morphogenesis it is logical to refer the cost to the worm as a whole. Estimated on the basis of oxygen consumption, the metabolic cost of morphogenesis during the second week of regeneration would be at least half as great as the cost of maintenance of the whole worm. Another estimate of a possible cost of morphogenesis is available from the data. Because of the starving condition of the worms, all substrates of metabolism in both intact and regenerating worms were necessarily derived from the older tissues; loss in weight should be proportional to the amount of the materials metabolized away. Materials which were transferred from the older tissues to the regenerant tissues should not enter into the determination, and thus loss in weight should be proportional to net rather than gross breakdown of substrates. It was found that during the same periods of time regenerating worms lost weight almost twice as rapidly as intact worms. On this second basis, morphogenesis has a metabolic cost nearly the same as the cost of maintenance of the entire worm. The cost estimated from weight loss is thus greater than that estimated from oxygen consumption. Probably not all of the breakdown of substrates is associated with oxygen consumption, and morphogenesis may have an additional cost in terms of activity of anaerobic metabolism or glycolysis.

Analysis of a metabolic cost of morphogenesis may be carried further by comparing the time course of rate of oxygen consumption with the time course of events in regeneration, especially the time course of the processes which were measurable in terms of segments per worm per day. During the period of highest rate of localization, oxygen consumption was only slightly above that of non-regenerating worms. During the earliest stages of differentiation oxygen consumption was almost precisely the same as in non-regenerating worms. This indicates that localization is not particularly costly in terms of aerobic metabolism, while initiation of differentiation appears to cost nothing. The evidence does not indicate that localization and early differentiation are independent of catabolism. Measurements of total oxygen consumption give no indication of the relative activities of different fractions of catabolism, nor do they indicate how much energy may be released through cellular oxidative systems which use some hydrogen acceptor other than molecular oxygen. (Evidence which indicates an "activity metabolism" of localization will be presented in a later paper.)

The peak in rate of oxygen consumption was found to coincide in time with the peak in rate of later differentiation. Furthermore, the earliest time of marked increase in oxygen consumption coincided with the time at which the later stage of differentiation began appearing, first at a low rate, then more rapidly, parallel with increase in rate of oxygen consumption. The marked increase in oxygen consumption in regenerating *T. tubifex* is associated with differentiation, or some process

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involved therein, and not with morphogenetic processes in general. It is suggested that differentiation is paid for in terms of metabolically released energy, and that the cost is high. As estimated from increase in oxygen consumption and in loss of weight, this cost must be at least half as great as the cost of maintenance of the entire worm.

Summary

1. Methods of measuring "rate of localization," "rate of early differentiation," and "rate of later differentiation," during posterior regeneration in *Tubifex tubifex* have been described.

2. A method of measuring oxygen consumption of individual worms has been described.

3. Oxygen consumption, as determined according to the above mentioned method and also according to the Warburg manometric method, has been found to proceed at a near normal rate during localization and early stages of differentiation in the first week of regeneration.

4. Markedly increased rate of oxygen consumption has been found associated with maximum rate of later differentiation during the second week of regeneration.

5. No significant cyanide-sensitive fraction of respiration was found at any stage of regeneration.

6. Worms in which regeneration had been inhibited by X-ray treatment showed no increase in oxygen consumption.

7. Loss of weight by the starving regenerating worms was found to be almost twice as great as by the intact worms.

8. The data have been discussed in terms of a metabolic cost of differentiation, which cost would be at least half as great as the metabolic cost of maintenance of the entire worm.

9. It is concluded that the marked increase in aerobic metabolism observed during regeneration in *T. tubifex* is associated with some process or processes involved in differentiation.

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THE EFFECT OF SEX AND AGE ON THE TEMPERATURE AT WHICH REVERSAL IN REACTION TO LIGHT IN ERISTALIS TENAX OCCURS

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Introduction

One of the most tantalizing phenomena facing the physiologist is the change of sign of reaction to light that occurs in many organisms. In this reversal an organism which is normally photopositive, moving toward the light, becomes negative and avoids the light, or a normally photonegative organism, moving away from the light, becomes positive. Some even consider this phenomenon "unapproachable," according to Maier and Schnierla (1935). Although this has claimed the attention of extremely able investigators for over 75 years, Holmes' statement (1916) holds good today. "The mechanisms involved are still unknown." The need for information concerning this problem is shown by the brevity of the paragraphs devoted to it by Wigglesworth (1939) and Heilbrunn (1943).

Reversal in reaction to light occurs "spontaneously" with no change in external conditions in Daphnia (Ewald, 1914; Clarke, 1932) and in Spondylomorum (Mast, 1918). It occurs rhythmically in some animals, according to Bohn (1905, 1907, 1909), Ewald (1914), and Warden, Jenkins, and Warner (1940), and in the course of normal development in others (Fraenkel and Gunn, 1940). For example, lobster larvae (Hadley, 1908) are positive for two days after hatching when they become negative and remain so until shortly before molting when they again become positive. They are negative in the early second stage and third stage but become positive before molting. In the fourth and later stages they are negative.

Various investigators maintain that a reversal of sign of the reaction to light can be produced in many organisms by the following changes: I. In the organism itself: metabolic rate, muscle tonus, water content, concentration of certain hormones, adaptation in sense organs, orientation to gravity, mode of locomotion (e.g., from swimming to crawling), mechanical stimulation, operations (such as removal of all or part of the wings or brain), training, and genetic constitution. II. In the immediate environment: temperature, food supply, background, light intensity (both gradual and rapid), wave length of light, osmotic pressure, oxygen pressure, hydrogen ion concentration, viscosity, and other changes produced by the addition of various inorganic and organic compounds.

Of all these methods none is more important than the use of alterations in temperature to produce changes in the sign of the reaction. An increase in temperature makes some photopositive organisms negative and some, that are negative, positive, while a decrease in temperature makes some positive organisms negative and some, that are negative, positive.

The animals and plants used in previous studies on the effect of temperature on reversal in reaction to light are as follows: swarm spores of *Haematococcus, Uloth-*

rix, and other algae (Strasburger, 1878); *Euglena, Volvox, Spondylomorum,* and other algae (Mast, 1911, 1918, 1927, 1932, 1936); the flagellate, *Chromulina* (Massart, 1891); *Rana clamata* (Torelle, 1903); *Arenicola* larvae (Kanda, 1919); *Polygordius* larvae (Loeb, 1893, 1905, 1906, 1918); *Lumbricus* and *Eisenia* (Prosser, 1934; Mast, 1936); *Cyclops, Cypris,* and a water spider (Mast, 1911); certain marine copepods (Loeb, 1893, 1905; Parker, 1901; Rose, 1929); *Daphnia* (Groom and Loeb, 1890; Loeb, 1906; Mast, 1911; Dice, 1914; Rose, 1929; Clarke, 1932); the copepod, *Leptodora* (Siedentop, 1930); *Artemia salina* (Bujor, 1911); *Balanus* nauplii (Groom and Loeb, 1890; Ewald, 1912; Rose, 1929); nauplii of the barnacle, *Chtamalus* (Rose, 1929); various amphipods (Phipps, 1915; Holmes, 1916); *Ranatra* (Holmes, 1905, 1916); *Notonecta* (Essenberg, 1915); the Mayfly nymphs, *Epeorus* and *Leptophlebia* (Allee and Stein, 1918); the beetle, *Anthrenus muscorum* (Janda, 1931); *Drosophila* (Carpenter, 1908); mosquito larvae, *Culex pipiens* (Miller, 1940); the tsetse fly, *Glossina morsitans,* and the stable fly, *Stomoxys calcitrans* (Jack and Williams, 1937).

The small number of species of insects tested furnishes no basis for Holmes' statement (1916), "In the insects reversal of the positive reaction is rather uncommon."

Because nothing is known about the effect of age and sex on the temperature at which reversal occurs in any organism, and because nothing whatever is known about reversal in *Eristalis tenax,* a study of reversal in reaction to light in this insect was made. For this work the drone fly proved as excellently adapted as it has for many other phases of physiological research.

In this paper are presented the results of a study of the effect of sex and age on the temperature at which reversal of reaction to light occurs. *Eristalis* at ordinary temperatures is highly photopositive. Preliminary experiments showed that between approximately 10°C. and 30°C. the flies crawl or fly directly toward a source of light. Outside these limits they are highly negative, moving directly away from a source of light. Since it was found impractical to study in detail the behavior of these flies at low temperatures but comparatively easy at high temperatures, this paper deals almost entirely with the latter.

Materials and Methods

The apparatus used (Fig. 1) consists of a box made of 6.3 mm. plywood (50 × 35 × 34 cm.). There are two main compartments, a light one, *A,* and a dark one, *B.* The light compartment is lined with white cardboard on the bottom, two sides and one end. The two compartments, 6.25 cm. deep, are separated by a wooden slide, *a,* painted white on the side toward the light compartment, which can be raised. Above the light compartment are two sliding glass panels, *b, c,* 6.3 cm. apart. A thermometer, *r* is inserted through a hole, *d* at the level of the white floor, *e.*

The dark compartment, *B,* has the same dimensions as the light compartment, but is lined with dull black cardboard and has a removable wooden cover, *f,* through the center of which a thermometer, *g,* is inserted. A glass window, *h* (3.8 × 12.5 cm.), covered by a removable shade on the outside, is at one end of this compartment.

Below the detachable floors, *i, j,* of each compartment the construction is identical. Five centimeters below these floors is a sheet of galvanized steel on which

rests a cardboard, *k*. Below the metal bottoms are two heat chambers, 12.5 cm. deep, *C, D*, lined with corrugated cardboard, *l*, and asbestos sheeting, *m*. The heat is supplied by two 100-watt Mazda lamps, *n*, with constant voltage, each lamp being wired separately.

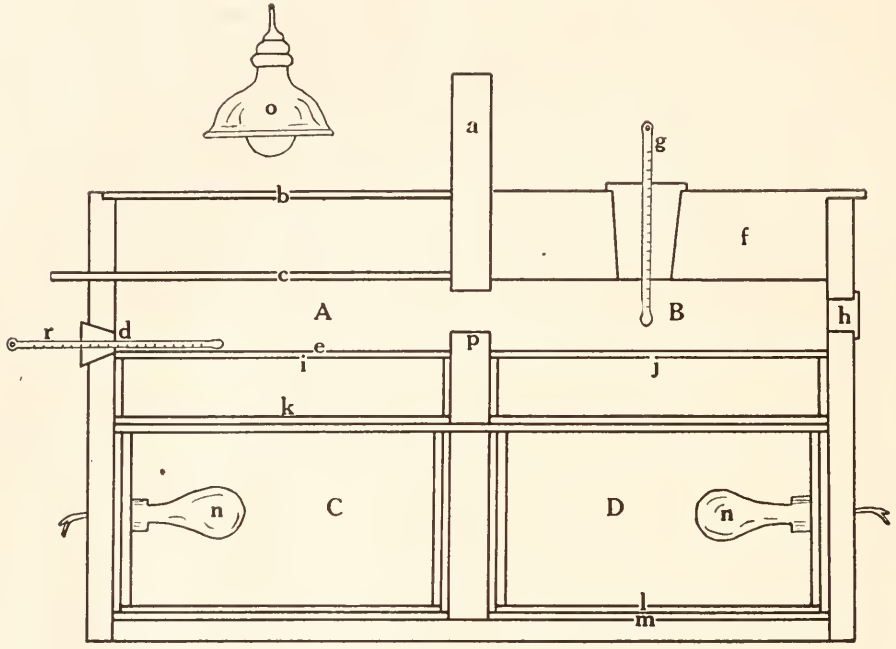

FIGURE. 1. Sectional view of the apparatus used. See text.

A 100-watt Mazda lamp, *o*, was suspended 81.25 cm. over the center of the white floor of the light compartment. The luminous intensity on this floor was 700 f.c., as recorded by a Weston exposure meter.

In these experiments only flies of known age and sex were used. They were raised in the laboratory according to the methods previously described (Dolley et al., 1937, p. 410). The exact temperature at which the flies became negative to light and went into the dark chamber was ascertained for six groups of flies whose sex and age are as follows:

1. Young males, 5–16 days.
2. Young females, 5–16 days.
3. Middle-aged males, 28–36 days.
4. Middle-aged females, 28–36 days.
5. Old males, 48–77 days.
6. Old females, 48–77 days.

Two hundred and seventy observations were made on members of each of these groups. No more than three were made on an individual insect and at least twenty-four hours elapsed between successive observations on the same fly.

All experiments were performed in a dark room. A typical experiment was made as follows: The temperature in the dark compartment was raised to between 35° C. and 37° C., that in the light compartment was raised to 28° C. Throughout the experiment the temperature in the dark compartment was seven to nine degrees higher than that in the light compartment. Ten to fifteen flies of the same age and sex, and with unclipped wings, were placed in the light compartment by sliding back the glass plates, *b, c*. These plates were replaced; the heating unit under the light chamber was turned on; and the center slide, *a*, was raised, making an opening (18 × 4 cm.) connecting the two chambers, *A, B*.

As the temperature in the light chamber rose the flies became restless, crawling and flying about. Soon individuals moved out of the light into the darkness of the dark chamber. A fly was considered to have reversed when it had passed completely beyond the center ridge, *p*. When this occurred the investigator recorded the temperature in the light compartment. The flies followed one another, and one by one entered the dark compartment. Frequently a fly returned to the light compartment after a few minutes in the dark, and then after a few seconds returned again to the dark compartment. Sometimes a given fly made three or four such successive reversals. The temperature at which the final reversal of a given fly took place was recorded and considered one observation. After approximately twenty-five minutes all the flies had reversed and entered the dark compartment. The organisms were then removed and those that had survived were placed in a cage with food and water. They were used for a maximum of two other experiments similar to that described above. An interval of at least twenty-four hours elapsed between successive tests on the same flies. About twenty-five per cent of the insects used in a given experiment did not survive their exposure to the high temperature of the dark compartment.

Results

The results obtained are given in Figure 2 and Table I. As is shown in this table, the mean temperatures in degrees centigrade at which the flies reversed are as follows: old males, 33.18 ± 0.09; old females, 33.822 ± 0.091; middle-aged males, 34.450 ± 0.088; middle-aged females, 35.06 ± 0.11; young males, 35.500 ± 0.099; young females, 36.106 ± 0.082. The standard deviations (Table I) are as follows: old males, 1.485 ± 0.064; old females, 1.489 ± 0.064; middle-aged males, 1.440 ± 0.062; middle-aged females, 1.742 ± 0.075; young males, 1.623 ± 0.070; young females, 1.318 ± 0.057.

Are the differences between the mean temperatures just given at which the two sexes reversed significant? According to Pearl (1940, p. 287), "the odds are 369.4 to 1 against the occurrence of a deviation in either the plus or minus direction as great or greater than 3 × S.E. These are long odds, and are conventionally regarded as amounting to practical certainty."

The differences between the means of the two sexes of the old, middle-aged and young flies are, respectively, 5 +, 4 +, and 4 + times the standard errors of the differences. This means that the odds against the occurrence from chance of these differences are, respectively, over 1,744,000; 15,770; and 15,770 to 1. It is evident that the differences between the means of the sexes in the above three age groups are clearly significant. Consequently, it is obvious that in each group the female flies reversed at a higher temperature than the male flies.

Age also has a definite effect on the temperature of reversal. This is shown when the observations on the individuals, both males and females, of the same age, are put together, as is done in Figure 2 and the lower portion of Table I. It is clear from this figure and table that the mean temperatures at which the old, middle-aged and young flies reversed are: 33.50 ± 0.07, 34.781 ± 0.071, and 35.786 ± 0.064, respectively. The differences between the means of the old and middle-aged, the old and young, and the middle-aged and young flies are, respectively, $12+$, $22+$, and $10+$ times the standard errors of the differences. Consequently, age is an important factor in determining the temperature at which *Eristalis* changes its reaction to light. The younger the fly, the higher the temperature at which reversal occurs.

TABLE 1

The effect of sex and age on the temperature at which reversal in reaction to light in Eristalis occurs. See text

Age	Sex	Mean temperature in degrees centigrade ± standard error	Standard deviation ± standard error
Old	male	33.18 ± 0.09	1.485 ± 0.064
	female	33.822 ± 0.091	1.489 ± 0.064
Middle-aged	male	34.450 ± 0.088	1.440 ± 0.062
	female	35.06 ± 0.11	1.742 ± 0.075
Young	male	35.500 ± 0.099	1.623 ± 0.070
	female	36.106 ± 0.082	1.318 ± 0.057
Old		33.50 ± 0.07	1.522 ± 0.046
Middle-aged		34.781 ± 0.071	1.652 ± 0.050
Young		35.786 ± 0.064	1.477 ± 0.045

It is patent that in the young flies the females with a standard deviation of 1.318 ± 0.057 showed less variation than did the males with a standard deviation of 1.623 ± 0.070; that in the middle-aged series the females showed greater variation than the males; that in the old flies the variation in the two sexes was about the same; and that between the three groups of different ages, each being composed of flies of both sexes, the young flies showed the least variation. The significance of the differences given above is not at present known, but it is true that the young flies were a more homogeneous group so far as age is concerned than were the old flies. The maximum difference in age between the members of the young series was eleven days, while the maximum difference in age between the members of the old series was twenty-eight days, a long period of time in comparison with the duration of life of *Eristalis*.

The reversals described above are unquestionably reversals to light and not to heat energy. The flies in going from the light compartment into the dark compart-

ment were not going from a region of high temperature to one of lower heat energy. The reading on the thermometer (Fig. 1, *r*) in the light compartment was compared with that of a black bulb thermometer made by coating a thermometer with lamp black. During the experiments the temperature as recorded by the latter was only 1.5 degrees higher than that recorded by the one used. Consequently, even if the flies had absorbed as much heat energy as did the black bulb thermometer, still the effective temperature in the dark compartment would have been from 5.5 to 7.5 degrees higher than that in the light compartment.

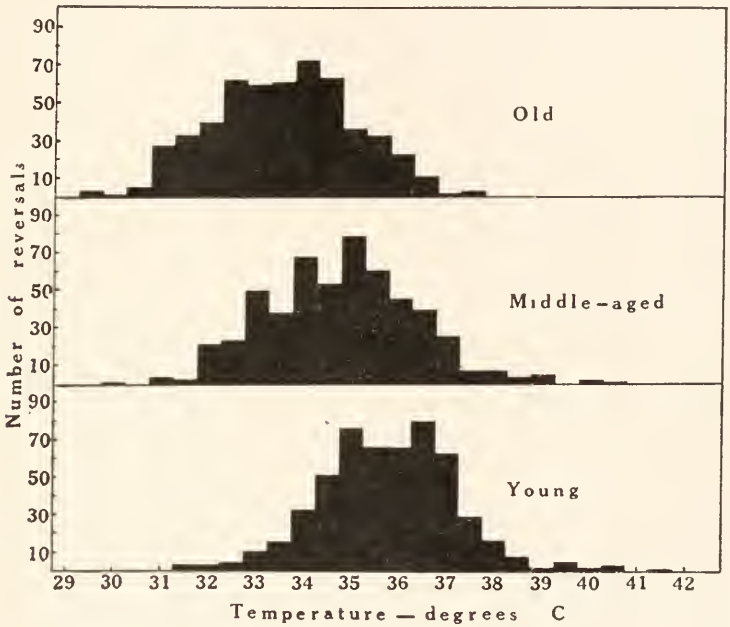

FIGURE 2. Histogram showing the effect of age upon the temperature at which reversal in reaction to light in *Eristalis* occurs. Note that the means of the reversals for the older flies are at lower temperatures than are the means for the younger ones. See Table I.

DISCUSSION

The results presented in this paper show that the temperature at which *Eristalis* reverses in its reaction to light is not correlated specifically with either a decrease or an increase in heat energy for it can be caused by both. Reversal in sign of reaction occurs both when the temperature is raised above a certain point and when it is lowered below approximately 10°C., as stated previously.

This conclusion is in harmony with the following: 1. Mast's contentions (1918) that a decrease in heat energy and an increase in light energy produce similar effects in photopositive euglenae and other algae, and (1936) that reversal in *Volvox* and *Euglena* is due to "internal changes" and "is not specifically correlated with the immediate environment"; 2. the conclusion of Phipps (1915) that reversal in certain amphipods is associated with changes in their "physiological states"; 3. that of Welsh (1930) that reversal in the water mite, *Unionicola*, "is probably a central nervous phenomenon"; 4. that of Washburn (1936, p. 208) that the "complex influ-

ence of external and internal conditions on phototropism" . . . "is based on innate factors"; 5. that of Allee and Stein (1918) that reversal in reaction to light in certain Mayfly nymphs is associated with either increase or decrease in metabolic rate; and 6. those of Jack and Williams (1937) that if the temperature is raised sufficiently high the normally photopositive reactions of the tsetse fly, *Glossina morsitans*, and the stable fly, *Stomoxys calcitrans*, become reversed, and that "the temperature at which the negative reaction develops appears to depend somewhat upon the flies' physiological conditions."

The conclusion stated above is not in harmony with the following: 1. Davenport's contention (1908, p. 200) that a "Diminution of temperature below the normal causes reversal of the normal response, elevation of the temperature to near the maximum accelerates the normal response"; 2. Maier and Schnierla's conclusion (1935) that the sign of the reaction to light in an organism "depends upon the characteristic metabolic condition of its species"; and 3. that of Holmes (1905) for *Ranatra* that any condition causing an increase in activity accentuates positive reactions and any quieting conditions make them negative.

The results presented in this paper show also that the temperature at which reversal occurs in *Eristalis* depends upon the resistance of the organism to the injurious effects of temperatures above and below the normal. The nature of this resistance is as yet unknown. Old flies are less able to endure the effects of abnormal temperatures than are young ones. Since the temperature at which reversal occurs in a given intensity of light is lower in old than in young flies, and in males than in females, it is probable that females are more resistant to the effects of temperatures outside the normal range than are males. This conclusion is confirmed by the results of work now in progress.

Reversal in response to light in *Eristalis* produced by changes in temperature is probably due to a different mechanism from that involved in the reversals occurring in normal development, which are doubtless associated with the development or degeneration of photosensory or other organs. As stated previously (Dolley and Haines, 1930), *Eristalis* larvae are highly positive to light for the first few hours after hatching. They then become negative to light and remain so until they pupate. The imagos are at first negative but soon become positive at ordinary temperatures, and remain so throughout their lives. Similar phenomena have been described for blowflies (Herms, 1911; Gross, 1913; Patten, 1916), *Amaroucium* larvae (Grave, 1920; Mast, 1921), and for other organisms. According to Pause (1918) Chironomus larvae are positive until they have formed haemoglobin when they become negative.

Summary

1. Over 1,620 observations were made on over 1,000 flies in ascertaining the temperature at which *Eristalis tenax* becomes negative to light.

2. In a luminous intensity of 700-foot candles *Eristalis* is highly photopositive within a temperature range between approximately 10° and 30°C. Outside these limits it is highly negative. Reversal of the photopositive reaction can be produced either by increase or decrease of the temperature.

3. In high temperatures the temperature at which *Eristalis* changes in its reaction to light depends on the sex of the flies. Females cease their positive reaction to light and become negative at a higher temperature than do males.

4. In high temperatures the temperature at which *Eristalis* changes in its reaction to light depends also on the age of the flies. The younger the fly, the higher the temperature at which it ceases its positive reaction to light and becomes negative.

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OBSERVATIONS ON THE REPRODUCTION OF THE SPINY DOGFISH, SQUALUS ACANTHIAS *

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The elasmobranch fishes offer an exceptional opportunity for studies of the physiology of reproduction. Within this old and diversified group of vertebrates many species are oviparous, others are ovoviviparous, and some are viviparous, thus indicating various degrees of physiological dependence of the young on the parent. Ranzi and his collaborators have made comparative studies of several representative species and their results and the observations of other workers have been presented in several reviews (see Needham, 1942, for discussion and references).

Our observations were made on two species of dogfish, *Squalus acanthias* (spiny dogfish) and *Mustelus canis* (smooth dogfish), common in the vicinity of Woods Hole, Massachusetts. The spiny dogfish is ovoviviparous and maintains its young free in the uterus while the smooth dogfish is viviparous and has young whose yolk sacs are modified into placental structures that are firmly attached to the uterine endometrium during the last two-thirds of pregnancy. Our chief interest was a study of the endocrines that might be associated with gestation in these two species of dogfish. This approach was quite different from the investigations of Ranzi and others which were primarily concerned with foetal nutrition. However, it was found that *Squalus acanthias* was not suitable for our experimental purposes. They could not withstand necessary surgical procedures nor could they live for long periods though kept in large, outdoor live-cars. Consequently, the results of our studies on *Squalus acanthias* were mostly observational. These are reported here while the results of our experiments on *Mustelus canis* will be published separately.

General Observations on the Reproductive Cycle

The different times at which *S. acanthias* is present at various points along the Atlantic coast indicate that it migrates northward in the spring and southward in the fall. It appears in Buzzards Bay and Vineyard Sound in late April or early May. It becomes very abundant during May but declines in number by the end of the month and is caught only infrequently toward the close of June. Correlated with its disappearance from the vicinity of Woods Hole is its arrival at successive points farther north, reaching the coastal waters of Newfoundland the last of June or early July. The migration southward apparently begins in September and spiny dogfish are again caught in large quantities by fishermen at Woods Hole in October and November as they pass by Cape Cod.

Our studies are based on animals obtained during the spring and fall migration, a period of about six months. Consequently we can only infer what changes might

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have occurred in the reproductive system during the winter and very early spring. However, we have examined several hundred spiny dogfish since our investigations were started in 1936 and believe that we have a fairly accurate account of the reproductive cycle and duration of pregnancy. Some of our observations are corroborations of certain details of the reproductive cycle well known to fishermen and collectors at Woods Hole, while others confirm the results of similar studies on *Squalus acanthias* by Hickling (1930) and Templeman (1944) in Newfoundland, and by Ford (1921), Ranzi (1934) and Popovici (1938) on *Acanthias vulgaris* as found in the English Channel, Mediterranian Sea, and Black Sea.

Most of this work was done at the Oceanographic Institution in Woods Hole and we wish to express our sincere thanks for generous laboratory accommodations. We also are indebted to Dr. H. B. Bigelow and Dr. W. C. Schroeder for many helpful suggestions and to Mr. James McInnis of the Marine Biological Laboratory, Supply Department, who made it possible for us to examine many hundreds of dogfish.

Uterine Young

The sexually mature females, when they arrive at Woods Hole during May, can be divided into two distinct groups depending on the stage of development of their embryos. For convenience we can designate these two periods of gestation as Stage *A* and Stage *C* (Fig. 1). Those females belonging to Stage *A* have ovulated recently while those of Stage *C* have foetuses 12 to 20 cm. in length. Females that are 80 cm. to a meter or more in length are pregnant almost without exception and all are invariably in either Stage *A* or Stage *C* of gestation. The pregnant females are not always equally divided between these two stages. In 1937 they were about equal but in 1938 there were about four in Stage *A* to one in Stage *C*.

The uterine ova of animals in Stage *A* are enclosed in a membranous envelope forming a structure commonly called a "candle." There is usually a candle in both the right and left horn of the uterus and each contains one to four ova. The average wet weight of 26 candles containing one to 4 ova (total of 65 ova) was 46.2 grams per ovum. The development of the embryos is about the same, each ovum having a blastoderm somewhat comparable to that of a hen's ovum at about 16 to 20 hours incubation.

The pups of Stage *C* have large pendent yolk sacs and are free in the uterine lumen. The range of variation in length is considerable (12 cm. to 20 cm.) and also there is a great difference in the size of the yolk sac, even among pups of the same length. Consequently there is a wide variation in the combined weights of the pups plus their yolk sacs ranging between 30 to 60 grams wet weight.

When the dogfish return to the vicinity of Cape Cod in October and November, the adult females again can be divided into two groups depending upon the condition of pregnancy. These two groups have been designated as Stage *B* and Stage *D* (Fig. 1). The ova of Stage *B*, though much further developed than those of Stage *A*, are yet enclosed in the candle membrane. The embryos are from 3.5 to 7.5 cm. long and are connected by an umbilical stalk about one inch in length to a large yolk sac about the size of the original ovum. The average wet weight of the embryo plus the yolk sac is about 40 grams.

Some of the females of the other group, Stage *D*, have given birth and consequently their uteri are empty while those that have not given birth have large pups

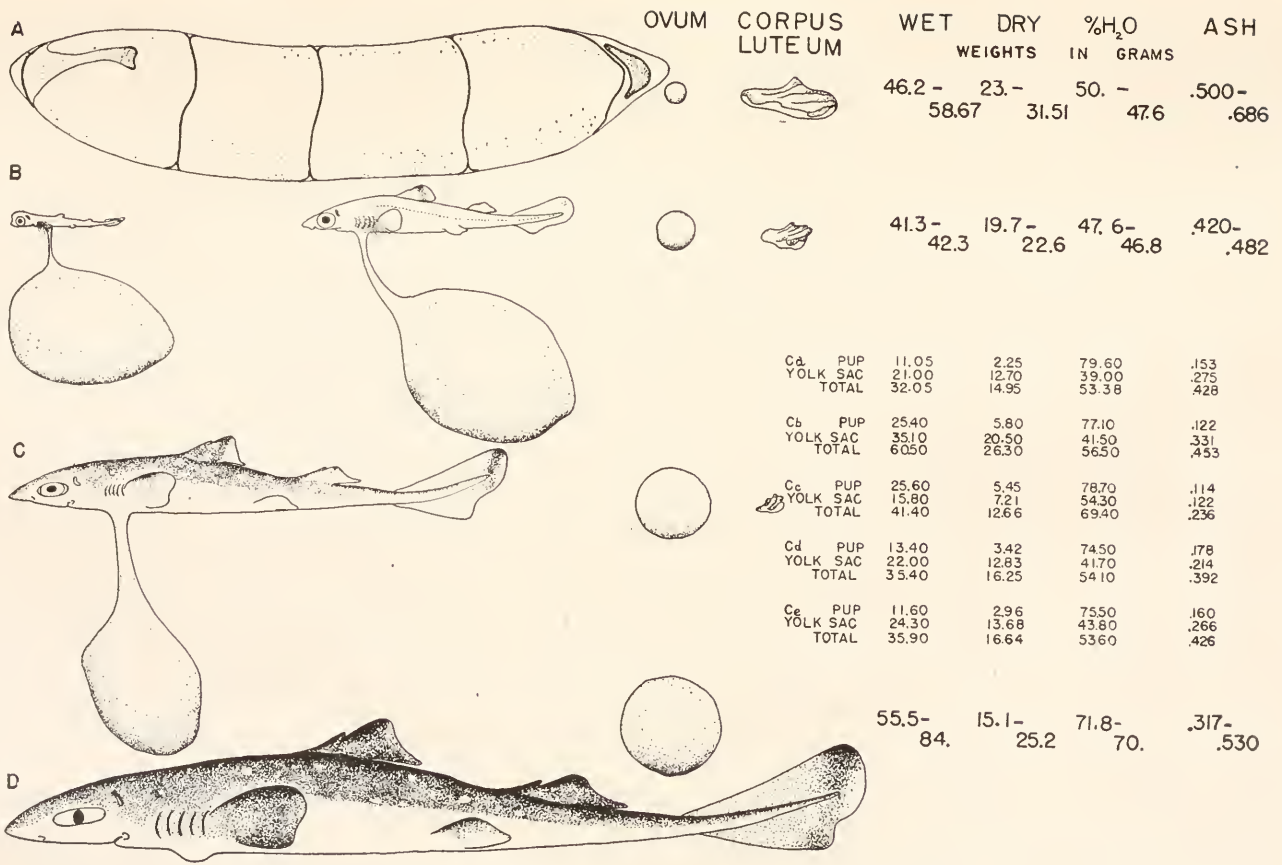

FIGURE 1. Comparison of the four stages of gestation in the spiny dogfish, *Squalus acanthias* as seen at Woods Hole, Massachusetts, in late April and May (Stages *A* and *C*), and October and November (Stages *B* and *D*). *Ovum.* Progressive increase in size of ovarian ova with the advancement of pregnancy. *Corpus luteum.* Gross appearance of the involuting corpora lutea. Drawings ¼ natural size.

that are 23 to 29 cm. in length. The yolk sac of some of these pups has been withdrawn into the body and the umbilicus is yet open while in others it is yet present though greatly reduced. The wet weight of such pups varies between 55 and 85 grams.

These four groups of uterine young obviously represent four different stages of gestation. When arranged in order of degree of development, which also agrees with the sequence of time, it readily is seen that the period of gestation is surprisingly long.

Ovarian Ova

When the ovaries of pregnant spiny dogfish representing the four stages of gestation are compared, they too show four corresponding stages in the development of the ovarian ova (Fig. 1). The early development of the embryos of animals of Stage *A* indicates that ovulation has occurred quite recently and consequently large ovarian ova are absent. Instead, there are numerous, usually 50 or more, small marble-white follicles the size of peas that contain ova usually weighing less than one gram. The embryos of Stage *B* are more advanced and likewise the ovarian ova. The largest eggs in the ovary are about the size of Concord grapes and have begun to take on a yellowish color due to the storage of yellow yolk.

The ovaries of Stage *C* females contain developing ova each of which usually weighs about 9 grams wet weight (variation 4 to 15 grams, average for 264 eggs, 9.1 grams). There are usually two or three such ova in each ovary, the total number never exceeding eight or nine, and they are not necessarily divided equally between the right and left side. They represent the next generation of ova that will be ovulated after the termination of the existing pregnancy and their average number is in close agreement with the average number of young found at one time in the uterus.

The average number of large ovarian ova for Stage *D* is about the same as that for Stage *C* but the eggs are considerably larger, being about 3 cm. in diameter and weighing between 30 and 40 grams. These weights are from two-thirds to three-fourths that of the candle ova of Stage *A* and strongly suggest that the eggs of the spiny dogfish at the time of ovulation probably average close to 50 grams. The fish of Stage *D* are seen at Woods Hole in October and November and the difference between the weight of the ovarian ova at that time and the ova in the candles of Stage *A* should give a fairly accurate estimation of the weight of the egg at ovulation, which probably occurs in February or March.

Thus it is seen that the ovaries of the four different groups show a progressive development of ovarian ova. Those of Stage *A* are small and numerous while in the succeeding three stages of gestation there is an increase in size and a decrease in number. It is, of course, quite common among the vertebrates for many small Graafian follicles to start developing even though only a very few ova are ovulated. In the dogfish as elsewhere the decrease in number of follicles is brought about by atresia. Most of such elimination must occur before Stage *C*, as we did not see large follicles that were undergoing atresia in either Stage *C* or *D*. Nor did we find large follicles in Stage *A* that had failed to ovulate and consequently were being resorbed.

Corpora Lutea

One of the interesting features associated with gestation in the dogfish is the formation of corpora lutea in the ruptured follicles following ovulation. Aside from the question of their physiological importance, the corpora lutea are useful in determining the time sequence of pregnancy as they undergo definite morphological changes as gestation proceeds.

The number of corpora lutea in the right and left ovary varies considerably. There may be six corpora in one ovary and none in the other or their distribution may be 4 and 4, 2 and 6, 3 and 3, 1 and 4, 2 and 4, etc. There is almost always an exact correlation between the number of corpora lutea and the number of embryos in the uterus. However, there is no correlation whatever between the number of corpora in any ovary and the number of embryos in the corresponding uterine horn on that side. The number of embryos in the candles of the right and left horns of the uterus tend to be evenly divided regardless of the unequal distribution of the corpora lutea between the ovaries. It seems that at ovulation an ovum may enter either horn of the uterus in a way probably similar to that described by Metten (1939) for *Scylliorhynus canicula*. However, in *S. acanthias* Templeman (1944) found, from a statistical study of several hundred animals that the number of young tends to be greater in the right uterus than in the left.

The corpora lutea of Stage *A* (Figs. 1, 2, 3) are large wrinkled sac-like structures 2 cm. or more across at the greatest diameter. Each is surrounded by a broad tissue space containing a clear fluid and consequently can be separated easily from the ovarian stroma and removed. Histological examination of such corpora shows a remarkable modification of the follicular wall which is thrown up in tall folds and drawn out in long lacy extensions into the follicular cavity. The luteal tissue seems to be derived entirely from the follicular granulosa.

The cells of the young corpus luteum first become very tall and have large vacuoles and irregular external cell borders (Fig. 2). They apparently have the ability to take up solid particles from the cavity of the ruptured follicle as occasionally a yolk granule is found in such cells. The ingestion of yolk by luteal cells is a common observation in the smooth dogfish and especially is this so during atresia of large follicles. Such activity of the corpus luteum will be described more fully in a subsequent report on the smooth dogfish.

The cells of the theca interna that are taken into the folds of the developing corpus do not take on a glandular appearance but contribute to the thin connective tissue framework that supports a rather generous supply of blood vessels. Cells derived from the granulosa are sharply separated from the underlying connective tissue structures.

There is considerable variation in the appearance of the granulosa-luteal cells of the corpora lutea of Stage *A* which seems to be correlated with age. The first reactions of the granulosa following rupture of the follicle and ovulation result in the development of the structure just described (Fig. 2). Other corpora are found, in animals of this stage, that seem to be older (Fig. 3). These are somewhat smaller and their walls thicker. The luteal cells have lost considerable vacuolation and are more tightly pressed into a folded epithelial-like lining of the luteal cavity.

Females with candle embryos taken in October and November, Stage *B*, have corpora lutea that are definitely older than those found in Stage *A*. This difference

in age is shown by a decrease in size of the luteal body and a reduction in size of the luteal cells which now have irregular shaped nuclei (Fig. 4). Also, those cells that lie next to the luteal cavity have become loosened and scattered through the luminal fluid, while the connective tissue of the folds has been modified into very conspicuous membranous partitions.

The corpora lutea of Stage *C* are smaller than those of Stage *B* and the luteal tissue is undergoing degeneration as shown by vacuolation and disintegration of the cells (Fig. 5). These changes must progress very slowly as the luteal bodies

FIGURE 2. Corpus luteum of Stage *A* showing changes that take place in the granulosa soon after ovulation. × 100.

can yet be distinguished in Stage *C* where they have been reduced to small yellow structures only 3 or 4 mm. in diameter. The luteal cells of these structures have lost most of their cytoplasm and about all that remains are small cells with picnotic nuclei scattered through the shrunken connective tissue framework of the original corpus luteum.

In Stage *D* very small, compact, yellow masses of tissue can be found that seem to be all that remains of the luteal bodies. Histologically, they are composed of small cells of irregular shapes with little cytoplasm and dense nuclei loosely distributed in the connective tissue skeleton of the old corpus luteum (Fig. 6).

These observations indicate that the corpora lutea in the spiny dogfish remain in the ovary for the better part of two years and during this time they undergo a progressive decrease in size and histological degeneration.

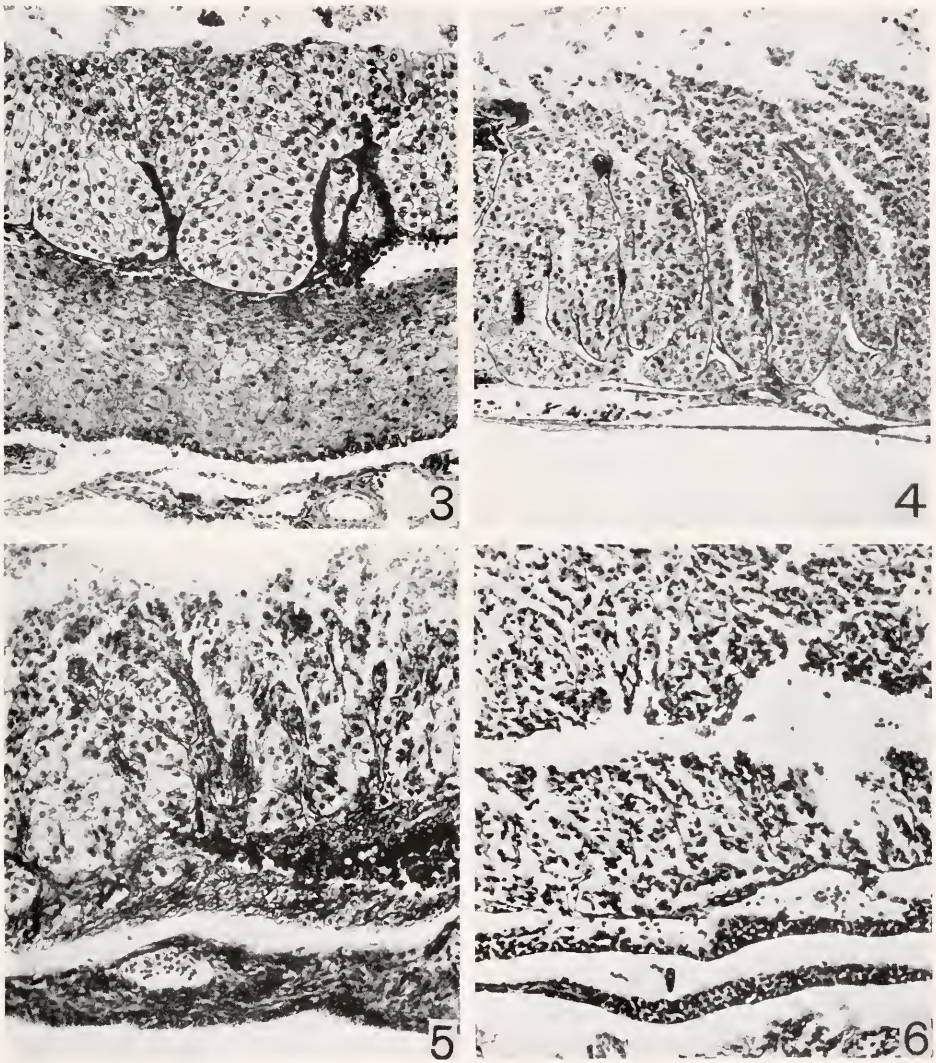

FIGURE 3. Corpus luteum of Stage *A* somewhat older than that shown in Figure 2. The granulosa luteal cells have lost their vacuoles, decreased in size and become organized into a folded multi-layered lining of the follicular cavity. × 100.

FIGURE 4. Corpus luteum of Stage *B*. The corpus luteum has decreased in size by at least one-half, and consequently the folds are more compressed. The luteal tissue is degenerating as indicated by a general decrease in size of the cells, small irregularly shaped nuclei and fraying with release of cells into the lumen.

The connective tissue inclusions between the folded granulosa luteal cells of the corpus luteum can be seen distinctly. × 100.

FIGURE 5. Corpus luteum of Stage *C*. Such corpora are about one year old and show advanced degenerative changes. × 100.

FIGURE 6. Corpus luteum of Stage *D*. Corpora of this stage are 18 to 20 months old and are reduced to small, yellow, compact bodies 2 to 3 mm. in diameter. × 100.

Nutrition of the Embryo

The embryos of *Squalus acanthias* develop free in the uterine lumen and possess no obviously specialized structures for obtaining nutritive materials from the uterine endometrium. Judging from their morphology they meet the requirements of a typical case of ovoviviparity. This and their extraordinarily long period of gestation of almost two years aroused interest in the problem of dependence or lack of dependence of the embryo on the parent as a source of nutrition. Consequently, representative material was collected from animals in the four stages of pregnancy we have described (Fig. 1) and data obtained on wet weight, dry weight, percentage of water and ash content. We have already mentioned the rather wide variations in size and weight of both ova and embryos so it is quite possible that our data are not sufficient in range and numbers to be considered as exact for the four stages of development represented, but we believe they show at least the general situation.

The ova of Stage *A* are surrounded by the thin membrane of the candle and, as they usually burst when this envelop is opened, it is extremely difficult to obtain weights of individual ova. Consequently, the whole candle was weighed, the small amount of fluid beneath the membrane at each end was withdrawn as completely as possible and the ova released into a dish by rupturing the envelop. After deducting the weight of the fluid and membrane the average weight of the ova was determined. Twenty-six candles, each containing one to four ova, when treated in this way gave an average weight of 46.2 grams per ovum. Seven ova, representing the upper limit of variation in weight, taken from two candles (3 in one and 4 in the other) of a large fish averaged 58.67 grams. This material was used for making the analyses shown in Figure 1.

In the study of Stage *B* an attempt was made to select embryos from the two extremes in the variation of development. At first, separate determinations were made for the embryo and yolk sac but for the smaller embryos the water content was so high and the amount of solids so small that it was thought a combined analysis would be better for the rather general purpose in mind. Such embryos are about 3.5 cm. long, weight 0.259 gm., and are 90 per cent water.

The pups of Stage *C* vary between 12 and 20 cm. in length, and they have yolk sacs of widely different sizes. From the standpoint of selecting material for analyses, this is complicated further by the fact that pups of the same length may have yolk sacs so different that one may be three times the weight of the other. Consequently, young that seemed to be in general representative samples of the upper and lower range in length were used for making the determinations shown in Figure 1.

Three small pups 12.5, 13, and 13.5 cm. long having an average wet weight of 11.05 gm. and dry weight of 2.25 gm. had an average total ash of 0.153 gm. Their yolk sacs averaged 21.0 gm. wet weight, 12.7 gm. dry weight and had an average ash content of 0.275 gm. The average combined ash of yolk sac and pup was 0.428 gm. A large pup 20.2 cm. long weighing 25.4 gm. wet weight, 5.8 gm. dry weight had an ash content of 0.122 gm. Its yolk sac weighed 35.1 gm. wet weight, 20.5 dry weight and had 0.331 gm. of ash. The combined weight of ash for the pup and yolk sac was 0.453 gm. A pup of about the same length (19 cm.) and weight (25.6 gm. wet weight, 5.45 gm. dry weight) but with a yolk sac only about half as large (15.8 gm. wet weight, 7.21 dry weight) had a combined ash content of 0.236 gm. (pup 0.114 gm., yolk sac 0.122 gm.).

These results have several points of interest. First the total ash of the young of this stage is not much different from that of Stage *B* although it is divided between the yolk sac and pup. Also the amount of ash is less than that found for the ova of Stage *A*. Another result that seems remarkable and is not understood is the relatively high ash content of the small pups.

The material used for the study of Stage *D* consisted of two large pups. The largest was 29 cm. long, weighed 84 gm. wet weight, 25.2 gm. dry weight, and had a total ash of 0.530 gm. The yolk sac was completely absorbed but the umbilicus was yet open (Fig. 1). The other was 24.5 cm. in length, weighed 55.5 gm. wet weight, 15.1 gm. dry weight and had a total ash of 0.3169 gm. This fish had a small yolk sac about 2 cm. in diameter (Fig. 1).

It seems obvious that the large pup was about ready to be born and equally obvious that the smaller pup never could have attained the size of the larger before parturition. Therefore there must be a wide difference in both length and weight of the young at birth. However, similar variations were found in all of the four stages of gestation that were studied, and these observations are in agreement with those made by Templeman. It seems reasonable to suspect that the size of a pup at birth may be correlated with the size of the ovum from which it came. If we make this assumption, then in our analyses, the large pups should be compared with the large ova and the small pups with the small ova. If the results for total ash are compared on this basis it is at once clear that the ash of the ovum exceeds that of the pup.

Therefore, it appears that the developing young of the spiny dogfish need not depend on the parent for inorganic matter. However, the presence of more inorganic matter in the egg than in the young at term does not preclude the possibility of a differential loss and absorption of mineral salts. Determination of the components of the ash would be helpful in answering this question but probably would not be conclusive.

Our data also indicate a loss of organic matter during development and an increase in water. When the ova of Stage *A* are compared with the mature pups of Stage D it is seen that the dry weight of the small ovum is 8.1 grams heavier than that of the small pup and the difference between the dry weights of the large ovum and large pup is 6.31 grams. The small pups also show a 75 per cent gain in water and the large pups a gain of 85.8 per cent.

These data were obtained from a few selected samples that we thought were representative of the four stages of gestation as seen at Woods Hole, Massachusetts. Therefore they should not be considered as being quantitatively exact but rather as showing the general trend of events taking place during development. It is definitely certain that the developing young obtain water from the parent, but whether they are entirely independent with regard to organic and inorganic nutrients is yet a question. However, these observations indicate that gestation in *Squalus acanthias* goes on under ovoviviparous conditions.

Discussion

Thus it is seen that the mature females caught at Woods Hole in the spring and fall are carrying young, almost without exception, and that they can be divided into four distinct groups on the basis of foetal development, size of ovarian ova and condi-

tion of the corpora lutea. These groups are so characteristic that no overlapping was found in the examination of several hundred fish. In fact it is extremely simple to determine the group to which a fish belongs by the gross appearance of the ovaries and uterine young.

It seems unmistakable that these four groups represent four stages of a gestation that extend over the better part of two years, probably 20 to 22 months. It is true that at present we can only surmise what takes place during the six months from November until May while the fish are on their southern migration. However, our records are fairly complete for the period between late April and November while these fish are in the waters along the New England coast.

When the fish arrive at Woods Hole in late April and May the embryos of the candle ova are small blastoderms and, judging by the slow rate of development, ovulation probably occurs in February or March. It is assumed that these fish (Stage A) as seen in April and May, are the same as those having candles in November (Stage *B*). Therefore, the difference between Stage *A* and Stage *B* represents the development that occurs in about the first six months of gestation, most of which is spent between Cape Cod and Newfoundland.

We also think that the fish in Stage *B* are those which, after their southern migration during the winter, return to Cape Cod the following May as fish in Stage *C*. During this period of about six months the uterine young increases in length from embryos of 3.5 to 7.5 cm. to pups of 12 to 20 cm.

These fish of Stage *C*, when next seen at Woods Hole in October and November, are in Stage *D* of gestation and a few have given birth to their young. The uterine young vary in length from about 24 to 29 cm. In some the yolk sacs are entirely withdrawn into the body while in others they are yet present but reduced to about 2 cm. in diameter. The indications are that the majority of births occur in late fall somewhere south of Woods Hole and that the pups are about 25 to 30 cm. in length when born.

Thus it seems that the only plausible explanation for these observations is that the spiny dogfish gives birth to young every other summer she visits the New England coast, and ovulates every other spring shortly before or soon after she starts on her northern migration. Furthermore, the female population is composed of two groups whose reproductive cycles are separated by about one year, and the four stages of gestation represent periods of approximately six months. Thus, each spring at Woods Hole the population includes only Stages *A* and *C* and in the fall only Stages *B* and *D*.

Our knowledge of the seasonal migration of *S. acanthias*, though fragmentary, tends to support the points just made concerning the relationships of the development of uterine young. Where the spiny dogfish spends the winter is a question that has not been answered but it is probably in deep water off the coast in the latitude of North Carolina. From there, judging from the observations of Bigelow and Welsh (1924), they arrive each spring in April almost simultaneously along the coast from Cape Lookout to Long Island. They usually appear at Woods Hole the last week of April or the first of May and are also found at about this season on Georges Bank. There seems to be a general migration northward from these points. Spiny dogfish do not appear in Massachusetts Bay before May, and Templeman (1944) reports their arrival along the coast of southern Newfoundland by

the middle of June and northern Newfoundland and Labrador by the second week of July.

Newfoundland and the southeastern coast of Labrador is the northern limit of migration. An additional point of interest is that by the time they become abundant in these waters they have almost entirely disappeared from around Cape Cod and points farther south. However, in Newfoundland, they arrive in June, become abundant in July and remain so until September or October. In contrast with this, they visit Woods Hole twice a year, in the spring and fall. Therefore, during their summer migration, which covers a period of about six months, approximately half of the time is spent in the general vicinity of Newfoundland. Judging from the observations made by Templeman the adult females are the first to arrive in the spring and apparently are the first to start south in the fall. In late November most of those caught were mature males, and immature males and females while only a few mature females were taken.

If the spiny dogfish make this round trip annually from their winter quarters, it is obvious that they must be capable of swimming great distances in a surprisingly short time. That they can do this is indicated also by Templeman's studies. He tagged 279 dogfish, mostly adult females, in the vicinity of St. John's Newfoundland between July 9th and 23rd and several were caught elsewhere in the same year. One of these, an adult female, was taken 132 days later close to Thatcher's Island Buoy, about 1,000 miles from the point where it was tagged. To travel such a distance this fish would have to average something like 7.6 miles per day even if it swam in a straight line which it is reasonable to believe it did not do. Thus it seems that at least the adult females are capable of covering what we believe is their migratory route.

A rather interesting deduction concerning migration also can be made by comparing the constitution of the population of spiny dogfish in Newfoundland and Woods Hole. Templeman states that the smallest dogfish caught was 58 cm. in length. At Woods Hole, small dogfish 40 to 50 cm. in length make up a considerable part of the general population. This indicates that these small fish, probably yearlings, do not reach the northern limit of the migratory range.

Certain differences in the stages of development of candle embryos and large uterine young of the fish examined by Templeman and ourselves also seem significant for determining migration. However, exact comparisons cannot be made as we did not make a large number of measurements that can be subjected to statistical treatment, but it is important to note extremes in size of candle embryos and large uterine young. The embryos of Stage *A* taken at Woods Hole in May were yet only blastodermic discs with an occasional exception in which a very small embryo could be seen. Of 139 comparable fish taken in Newfoundland in July, 104 had eggs showing blastodermic discs while the others had small embryos, the largest found in two animals being 1.8 and 2.0 cm. respectively. No embryos comparable to these largest ones have been seen at Woods Hole in May, and the fact that the great majority were yet blastodermic discs in July indicates that the rate of development is slow. However, in August, Templeman found only one fish in a group of 42 with eggs having blastodermic discs.

During the summer in Newfoundland growth of these candle embryos proceeds until by October and November they are from 2 to 4 cm. in length. However, by

the first of October most of the large females have left Newfoundland and it is of interest that at this time they become abundant around Cape Cod and constitute the group we have designated as being in Stage *B* of gestation. Templeman's data based on measurements of the embryos of nine fish during October and November fall well within the range of those for our Stage *B*. The only exception being that we found some embryos that were about 8 cm. in length but at the same time we had an opportunity to examine a much larger number.

Dogfish with pups having large yolk sacs (Stage *C*) taken at Woods Hole in May are comparable with those caught in Newfoundland in July. The length of the pups in Newfoundland varied from 12 to 18 cm. (average 15.59 cm.). A few at Woods Hole were 20 cm. in length, but this is probably an unimportant variation. There is also considerable variation in the size of the yolk sac. These differences in length and size of yolk sac, it seems, do not indicate necessarily a corresponding difference in age but rather is correlated with the size of the ovum at ovulation.

Such young, during the stay of the parent in Newfoundland from July to November, grew in length from an average of 15.59 cm. to 21.43 cm. The average length of the young in November is essentially the same as that for similar young taken at Woods Hole during the same month. However, there were certain interesting exceptions. The largest pups among those measured by Templeman were 25.5 cm. in length, while our largest measured 29.00 cm. The yolk sacs of these large pups had been absorbed almost completely while those of others somewhat smaller were reduced in diameter to about 1.75 to 2.0 cm. There were also a few large females with empty uteri. The large ovarian ova and uteri of these animals indicated that they had given birth to their young just recently. It seems probable that the great majority of births occur at a later time somewhere south of Woods Hole, but in a few instances young are released during the fall migration southward.

Conclusions

The spiny dogfish, *Squalus acanthias,* of the Atlantic coast migrate northward in the spring and southward in the fall. They are present at Woods Hole, Massachusetts, from late April to early June and again in October and November. The adult females when they appear in the spring, have uterine young which are either early embryos enclosed in membranous capsules ("candles") or more advanced "pups" 12 to 20 cm. in length, with large pendent yolk sacs, and free in the uterine lumen. Adult females, when seen in the fall, have candle embryos 3.5 to 7.5 cm. long or large pups 25 to 29 cm. in length, whose yolk sacs are greatly reduced or completely absorbed, while a few females have given birth to their young. Thus four distinct stages of gestation are seen when the fish are divided on the basis of development of uterine young.

The gestation period apparently covers about 20 to 22 months and a female gives birth every other year. This opinion is supported by the progressive advancement in development of the young in the four stages of pregnancy. Also, correlated with this is a gradual involution of the corpora lutea and a corresponding increase in size of the ovarian ova which will be ovulated following the conclusion of the existing pregnancy.

Analysis of candle ova and pups of the four stages of gestation show an increase in water and a decrease in both organic and inorganic matter as indicated by dry

weights and content of ash. Thus it seems that gestation in the spiny dogfish should be considered as being ovoviviparous.

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THE CULTURE OF VOLVOX AUREUS EHRENBERG

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Introduction

A number of methods for the culture of *Volvox* are described in the literature, but none of these has been consistently successful in our laboratory. Other investigators have had a similar experience. S. O. Mast (personal communication) and his students have tried these methods time and again over a period of years without securing permanent cultures.

Hartmann (1921) was unsuccessful in culturing *Volvox* although he was able to maintain cultures of *Eudorina elegans* for many months. Knoke's many experiments (1924) over a two-year period were likewise without positive results. After extensive analyses of natural waters containing *Volvox* in large numbers, Uspenski and Uspenskaja (1925) devised an inorganic salt solution (based on Knop's medium) in which *Volvox aureus* Ehrenberg and *V. globator* Linnaeus were cultured bacterium-free for periods of fifteen and four months respectively. These workers showed that a favorable concentration (0.5–1.0 mg./l.) of iron was a decisive factor in the production of healthy cultures, a deficiency of iron resulting in gradual deterioration, an excess of iron producing a poisoning of the *Volvox* colonies. Soil extract or decoction was used by Mainx (1929) who secured rich permanent cultures of *V. aureus* far superior to those obtained with synthetic solutions, including that of Uspenski and Uspenskaja. Pringsheim (1930) reached similar results in experiments with *V. globator* and *V. aureus*, but he likewise was unable to maintain these organisms for any length of time in the Uspenski medium where their growth was slower and of shorter duration than in soil extract medium. Lefévre (1932) states that he secured a clone culture of *V. aureus* in a nutritive solution (derived from that of Czurda) consisting largely of inorganic salts in pond water to which a few fragments of *Sphagnum* were added. About 300 descendant colonies were produced in some ten days, but he does not say whether this clone was maintained for any appreciable length of time. Mixed cultures of Volvocales and other plankton species, which included *V. aureus*, *Pandorina morum* Bory, and *Eudorina elegans* Ehrenberg, were maintained for many months.

Johansen (1940) lists six solutions which produce luxuriant growth of *Volvox*, *Gonium*, *Pandorina*, and *Eudorina*, namely certain soil solutions; 0.05 per cent Benecke's and 0.05 per cent Knop's solutions; and 1.5 per cent Detmer's agar (apparently after Bold, 1936). He also gives a medium which is identical in composition with that of Uspenski.

It would appear from the foregoing that the continuous culture of *Volvox* is a relatively simple and exact matter, and that a variety of media are available for this

[1] I gratefully acknowledge the friendly interest and encouragement in this work shown by the late Dr. S. O. Mast, emeritus professor of zoology, The Johns Hopkins University.

purpose. Nevertheless, preliminary experiments indicated that this is not the case. Soil decoctions vary widely in composition with the soil used, and inorganic salt solutions may not give rich, permanent cultures.

The purpose of the present paper is threefold: (1) to describe a culture method for *V. aureus* which has given consistent results for eighteen months in the continuous culture of the organism; (2) to reduce this method to one which is readily duplicable; and (3) to indicate by experiments the probable complexity of the nutrition of this organism.

Materials and Methods

V. aureus was secured from a pond on the property of the Carolina Biological Supply Company near Elon College, North Carolina, where it occurs throughout the year, appearing intermittently in large numbers.

In all of the experiments ordinary finger bowls (4½ inches ×2 inches) were employed as culture dishes. Two hundred cc. of medium were dispensed in each of the bowls which were then placed in a hot air oven and pasteurized. Inoculations were made after the medium had cooled to room temperature (approximately 21° C.). All cultures were illuminated by the light through a west window, some direct sunlight falling on them during part of the afternoon. Throughout the winter months the low light intensity was supplemented by radiation from a Sylvania fluorescent lamp (Type HF–150S).

Inoculations were made with ordinary medicine droppers which had been previously boiled. No attempt was made to exclude bacteria from the cultures, which however showed little evidence of bacterial action. All other possible contaminants such as protozoa and algae were absent.

The salts used in the preparation of inorganic media were either Baker's Analyzed or Merck's Reagent. The peptone and beef extract were Difco, the creatine was Pfanstiehl cp., the lactic acid was Baker's Analyzed, and the uric acid was Coleman and Bell's Reagent. In all cases stock solutions were made up and then diluted to proper concentration in the preparation of media.

The commercial fishmeal (commonly used as fertilizer) was obtained from Ballard Brothers, Willis Wharf, Virginia. The spring water used in most of the experiments was secured from a surface spring on the property of the Carolina Biological Supply Company. It is referred to as local spring water. The Huckleberry spring water came from a spring by that name near Durham, North Carolina. Distilled water was supplied by a Stokes Automatic Water Still.

Experiments and Results

Experiments with inorganic salt solutions

Modified Knop's medium as recommended by Bold (1936) and Johansen (1940) was prepared and dispensed into finger bowls. Five cultures were inoculated with several hundred colonies of *Volvox*. The cultures were examined daily, but no growth or reproduction was evident. The colonies gradually lost their rich green color and fell to the bottom of the bowl where they remained motionless, and finally deteriorated. Although the experiment was repeated a number of times the result was always the same.

BIOLOGICAL LAB WOODS HOLE

Since Uspenski and Uspenskaja (1925) cultivated *V. aureus* and *V. globator* in an inorganic medium continuously over periods of fifteen and four months respectively, attempts were made to secure similar results with their medium. The composition is as follows:

KNO_3	0.025 gm.
$MgSO_4$	0.025 gm.
$Ca(NO_3)_2$	0.100 gm.
KH_2PO_4	0.025 gm.
K_2CO_3	0.0345 gm.
$Fe_2(SO_4)_3$	0.00125 gm. (added every 10 days)
Distilled water up to 1,000 cc.	

Whereas Uspenski and Uspenskaja worked with bacterium-free cultures and used Leningrad glass culture dishes, the present experiments were conducted in ordinary glass finger bowls containing pasteurized medium. The initial pH was found to be 7.6 which is in exact agreement with these authors.

Five cultures were prepared. Each was inoculated with a single large *Volvox* colony bearing eight daughter colonies. Only one culture showed any evidence of growth and reproduction about a hundred pale green colonies being evident after several weeks. These were permitted to concentrate at one side of the bowl, removed with a medicine dropper, and used as inoculum for five subcultures. Observation over a period of four weeks showed no evidence of growth and reproduction. Gradually the colonies lost their motility, became paler in color, and finally disintegrated. Although the experiment was repeated a number of times, the results were negative.

It was noted that the ferric sulfate had a tendency to be precipitated in some of the cultures shortly after they were pasteurized. Believing that failure of the experiment might be due to a lack of the iron salt, it was repeated with the exception that $FeCl_3$ was used instead of $Fe_2(SO_4)_3$; but, although the former salt remained in solution somewhat better than the latter, the end result was the same.

Experiments with media containing organic materials

Soil decoctions. Several samples of local garden soils were obtained, extracted, and diluted according to the methods of Mainx (1929) and Pringsheim (1930). Although numerous attempts with varied concentrations of soil extract medium were made to secure cultures comparable to those described by these workers, success was not realized. In most instances the organisms rapidly deteriorated and died. In several cases multiplication occurred for a time, but efforts to maintain these cultures through subcultivation failed.

Peptone, beef extract, milk, and urine. Various concentrations of these substances in spring water ranging from 5 mg./l. to 200 mg./l. were tested without securing growth and reproduction for more than a week or so. The first cultures of *Volvox* in beef extract medium (5 mg./l.) exhibited an astounding rate of multiplication for several weeks, thousands of colonies being produced. Subcultures, however, were poorer. Gradually the colonies became paler in color, and after six weeks the third set of subcultures died out. Some evidence of growth was secured in peptone medium (5 to 10 mg./l) but *Volvox* colonies quickly deteriorated in all-concentrations of urine and milk media, in which a considerable growth of bacteria

was evident. Even though all of the culture media contained $FeCl_3 \cdot 6H_2O$ (0.5 cc. 1 per cent sol./l.), this failed to prevent the cultures from dying out.

Commercial fishmeal. An extract of commercial fishmeal was prepared by adding 200 mg. of fishmeal to a liter of spring water and heating to 80–90° C. The mixture was shaken well and filtered (Reeve filter paper, No. 201). The slightly straw-colored filtrate was dispensed in five finger bowls which were then pasteurized. Several hundred *Volvox* were inoculated into each of these. After two weeks one of the cultures was teeming with colonies. Five subcultures from it produced thousands of the organisms. Subcultivation was continued every two weeks, but after six weeks all of the descendant cultures suddenly died out.

This experiment was repeated in exactly the same way except that the cultures were aerated daily by bubbling air through the medium, but the end result was the same.

Recalling that Uspenski and Uspenskaja (1925) had emphasized the importance of iron in the nutrition of *Volvox,* fishmeal extract was prepared as in the previous experiments. To each liter 0.5 cc. of a one per cent solution of $FeCl_3 \cdot 6H_2O$ was added. This gave a concentration of the salt of 5 mg./l. or of 1.03 mg. Fe/l. This amount of Fe is slightly greater than the optimum (0.5–1.0 mg./l.) given by Uspenski and Uspenskaja, but it was felt that some of the Fe would be bound by organic substances in the medium and so reduce the available supply. The amount of Fe remaining unbound would be within the optimal range.

Five cultures were set up and inoculated from a clone culture that had been established in fishmeal extract several weeks earlier. This culture contained no iron other than that already present in the spring water used in its preparation.

In approximately two weeks excellent cultures resulted. From these cultures others were established which were in no way inferior. Thus, this clone of *Volvox* has been maintained in continuous culture with undiminished vigor for a period of eighteen months.

Following the recommendation of Uspenski and Uspenskaja, only half as much iron (¼ cc. of a one per cent solution of $FeCl_3 \cdot 6H_2O$) was added in winter as in summer. Since the initial concentration of iron was rather high, iron was not added every ten days in summer and once a month in winter as they suggested. The initial pH of the fishmeal medium was consistently 7.6. Over a three-week culture period it was found to fluctuate around this value.

Fishmeal medium, prior to the addition of ferric chloride, is faintly straw colored but clear. After twenty-four hours it becomes slightly cloudy, perhaps through bacterial action. No membrane forms at the surface, however, and in a few days the medium becomes clear.

No attempt has been made to count the total number of colonies in a single culture, but the number surely runs up to several thousand. Cultures reach a maximum population in from two to three weeks depending largely on variations in light intensity and temperature. Over a period of eighteen months reproduction has been asexual; sexual reproduction has not been observed.

Extracts of different strengths in which the amount of fishmeal varied between 50 and 1,000 mg./l. were tested, but 200 mg./l. seemed to give the largest populations of *Volvox.* Therefore, all stock cultures were maintained in media prepared with that amount of fishmeal.

Composition of fishmeal extract

Since *V. aureus* flourished in a medium prepared from spring water, fishmeal, and ferric chloride, it seemed desirable to determine the essential components with a view toward simplification. In this connection the following questions are pertinent: Can a spring water of known chemical composition be substituted for the local spring water? Can spring water be replaced by either tap water or distilled water? Can Uspenski's medium be used in place of spring water? Are the inorganic salts alone in fishmeal, along with those in spring water, sufficient to support the culture of *Volvox?* Does the fishmeal extract contain an appreciable amount of protein, and if so, how important is this in the medium? How important are other extractives?

Water component of fishmeal extract. Seven lots of five cultures were prepared in which the following media were used:

1. Fishmeal + local spring water + $FeCl_3 \cdot 6H_2O$
2. Fishmeal + Huckleberry spring water + $FeCl_3 \cdot 6H_2O$
3. Fishmeal + tap water + $FeCl_3 \cdot 6H_2O$
4. Fishmeal + distilled water + $FeCl_3 \cdot 6H_2O$
5. Fishmeal + Uspenski medium
6. Fishmeal + Uspenski medium ($FeCl_3 \cdot 6H_2O$ used instead of $Fe_2(SO_4)_3$)
7. Local spring water + $FeCl_3 \cdot 6H_2O$

In all cases 200 mg. of fishmeal and ½ cc. of a one per cent solution of $FeCl_3 \cdot 6H_2O$ were used.

Each culture was inoculated with ten large *Volvox* colonies of approximately equal size, each colony containing eight daughter colonies.

Volvox flourished in media 1 and 2, but little or no growth occurred in 3, 5, 6, and 7. Large numbers of colonies were produced in medium 4. These were pale green in color, becoming paler with each subcultivation, and finally were moribund after four transfers (68 days). Cultures in media 1 and 2 were maintained for many months and were in a flourishing state when finally discarded. Repetition of the experiment gave substantially the same results.

This experiment clearly shows that the chemical composition of the water used in fishmeal medium is very important in the culture of *Volvox*. Tap water may not be nutritively deficient but toxic, for it has been observed that paramecium, hydra, and other invertebrates do not survive long in the local unchlorinated, artesian water. Distilled water is obviously deficient in certain essential salts, although the lack is not felt for some time. Huckleberry spring water can be substituted for the local spring water since it can supply these salts in adequate amounts. It is interesting to note that the Uspenski medium, prepared to include either ferric sulfate or ferric chloride, failed to support growth and reproduction of *Volvox*, even when fortified with the extractives of fishmeal. Finally, fishmeal supplies important nutritive substances without which cultures of *Volvox* soon perish.

Fishmeal component. Obviously fishmeal is a very complex material. Therefore, no extensive series of experiments was contemplated to determine what substances in fishmeal are so vital in the nutrition of *Volvox*. The following experiment was performed to make clear what class or classes of substances (inorganic

salts, proteins, other extractives) in fishmeal contribute to the growth and reproduction of *Volvox*. Four culture media were prepared according to the schema:

1. Fishmeal ash + local spring water + $FeCl_3 \cdot 6H_2O$
2. Fishmeal + cold local spring water + $FeCl_3 \cdot 6H_2O$
3. Fishmeal residue + hot local spring water + $FeCl_3 \cdot 6H_2O$
4. Washed fishmeal + local spring water + $FeCl_3 \cdot 6H_2O$

One gram of fishmeal was completely incinerated in a chemically clean crucible and the resulting ash shaken well with one liter of spring water. The mixture was heated to 80–90° C. and filtered while hot. Two hundred cc. of this solution were diluted to one liter with spring water in order to secure an inorganic salt concentration comparable to that of regular fishmeal extract.

Another gram of fishmeal was added to a liter of spring water, shaken well, and filtered. Two hundred cc. of the filtrate were diluted with spring water to one liter to give a solution of cold-water extractives approximating the concentration of these substances in regular fishmeal extract.

The residue on the filter paper was added to a liter of spring water and heated to 80–90° C. and filtered while hot. Two hundred cc. of the filtrate were diluted with spring water to one liter. Theoretically this solution contained hot-water extractives roughly equivalent to their concentration in regular fishmeal extract.

The residue on the filter paper was dried and weighed. It had lost one-fourth of its weight. This amount (0.25 gm.) was shaken well with a liter of spring water to form a suspension.

One-half cc. of a one per cent solution of $FeCl_3 \cdot 6H_2O$ was added to each liter of culture medium. Five cultures were prepared from each of the four media, pasteurized, and allowed to cool to room temperature. Each culture was inoculated with five large *Volvox* colonies, every one of which contained eight daughter colonies in about the same state of development.

Examination of the cultures over a period of three weeks gave no evidence of growth in media 1, 3, and 4. Medium 2 on the other hand gave excellent cultures which were in no respect inferior to stock cultures maintained on regular fishmeal extract. Subcultures, which were carried for several months, showed no sign of deterioration whatsoever and probably could have been maintained indefinitely.

This experiment demonstrates, therefore, that the readily soluble cold-water extractives of fishmeal complement the inorganic salts of spring water and the ferric chloride to produce a nutritionally complete medium; that the soluble salts in fishmeal ash medium do not meet all of the growth requirements of *Volvox;* and that hot-water extractives and proteins of fishmeal have little or no importance in the culture of *Volvox*.

It might be supposed that proteins would be present in the cold-water extract fraction of fishmeal, but the usual tests for protein were negative.

Fish extracts

Commercial fishmeal consists largely of the pulverized remains of marine fishes, and therefore contains all of the chemical constituents of bone, muscle, skin, viscera, etc. The question as to what part of a fish, if any one part, provided the important nutritive substances in the culture of *Volvox* was investigated as follows:

Several butter-fish (*Poronotus triacanthus* Peck) were secured at a local fish market. These were washed in distilled water. Small quantities (about 10 gm.) of skin, muscle, and bone (vertebral column) were removed very carefully in order not to include any of the adjacent tissues. The three portions were placed in a small amount of distilled water in three separate flasks and boiled until practically all of the water had evaporated. They were then dried on filter paper in a dry air oven. One-half of each portion was separately shaken with ether from time to time for a period of four hours in order to remove lipoids, after which treatment the ether was removed by filtration. The skin, muscle, and bone residues were dried on filter paper for forty-eight hours. The remaining half of each portion of skin, muscle, and bone received no further treatment.

Two hundred mg. each of skin, skin extracted with ether, muscle, muscle extracted with ether, bone, and bone extracted with ether were separately heated with one liter of spring water to 80 – 90° C. and filtered while hot. One-half cc. of a one per cent solution of $FeCl_3 \cdot 6H_2O$ was added to each liter of filtrate. Five cultures were prepared from each type of filtrate and inoculated with numerous *Volvox*.

The results are summarized below:

1. Skin medium—colonies died within a few days
2. Extracted skin medium—colonies died within a few days
3. Muscle medium—good cultures; subcultured for three months
4. Extracted muscle medium—excellent cultures; subcultured for three months
5. Bone medium—colonies died within three weeks
6. Extracted bone medium—some growth and reproduction; subcultures unsuccessful

The experiment was repeated using Huckleberry spring water instead of the local spring water. The results were essentially the same with the exception that colonies in the extracted bone medium died out within a week.

The experiment shows that, of the three tissues tested, muscle alone contains an adequate quantity of the substances so vital to the continuous culture of *Volvox*. Furthermore, the conclusion is reached that the lipoids are of no value to *Volvox;* on the contrary they appear to exert a depressing influence as is indicated by the larger populations of extracted muscle medium cultures.

No attempt is made here to deny that connective tissue associated with fish muscle may contribute as much or more than muscle to the nutrition of *Volvox*. It would be next to impossible to separate the two tissues to determine this point. However, in experiments which follow the assumption is made that it is the muscle that is important and not the connective tissue.

Since the butterfish is marine and the associated salts of sea water might conceivably have something to do with the results obtained, experiments were carried out with the fresh-water sunfish (*Lepomis gibbosus* Linnaeus) in order to check this presumption. The ether-extracted, powdered muscle gave flourishing cultures which were maintained in subculture for three months and then discontinued. Doubtless muscle tissue from other fishes could have been used with similar results.

Experiments with some organic constituents of fish muscle

Whatever the nature of the constituents of fish muscle that are so important in the culture of *Volvox,* it is clear from the foregoing experiments that the proteins,

lipoids, and inorganic salts are unnecessary. This leaves carbohydrates (glycogen, etc.), nitrogenous extractives (creatine, urea, uric acid, etc.), non-nitrogenous extractives (lactic acid, inosite, etc.), pigments, enzymes, and growth-promoting factors, and perhaps other substances to be considered. It seemed most likely that one or more of the nitrogenous and non-nitrogenous extractives might support the continuous culture of *Volvox*. Therefore, experiments were undertaken in which lactic acid, urea, creatine, and uric acid were tested singly and in combination in various concentrations from 5 mg./l. to 100 mg./l. of local spring water, the usual amount of ferric chloride being added to all media. None of the cultures, however, was successful. In several instances adult colonies released daughter colonies, but these gradually lost their healthy green color and soon deteriorated. In a medium composed of urea, peptone, and lactic acid (5 mg./l. each) as many as three generations were produced before death occurred.

A duplicable culture medium for Volvox aureus

An analysis of Huckleberry spring water by D. M. Pace some fifteen years ago (personal communication) showed it to have the following inorganic composition:

Na_2SiO_3	12 mg./l.
NaCl	12 mg./l.
Na_2SO_4	6 mg./l.
$CaCl_2$	6.5 mg./l.
$MgCl_2$	3.5 mg./l.
$FeCl_3$	2–3 mg./l.

An artificial spring water was made up according to the above formula except that sufficient ferric chloride solution was added to give a concentration of this salt of 5 mg./l. Using ether-extracted, powdered sunfish muscle (200 mg./l.), culture medium was prepared according to the same method previously described for the fishmeal and fish muscle media. Cultures of *Volvox* have been maintained on this medium in a flourishing state for a period of four months, showing no evidence of decrease in vitality. These cultures were in every respect equal to cultures of the organism secured with commercial fishmeal extract.

Although fishmeals may vary somewhat in composition depending on their source and mode of manufacture, the powdered sunfish muscle prepared as herein described may be expected to have a constant composition wherever prepared. This method for the culture of *V. aureus*, therefore, is readily duplicable.

Discussion

Results of the foregoing experiments fail to confirm those of Uspenski and Uspenskaja (1925), Bold (1936), and Johansen (1940) who have cultured *Volvox* in inorganic salt solutions in the absence of organic matter. The findings, however, are in agreement with those of S. O. Mast (personal communication) and his students who were unable after many experiments to secure permanent cultures of *Volvox* in Uspenski's, Knop's, and other synthetic media. Pringsheim (1930) was evidently unable to maintain *V. aureus* and *V. globator* for any length of time in the Uspenski medium.

It is difficult to believe that failure to culture *Volvox* in the Uspenski medium was a result of impurities in the salts or the kind of glassware used in the present experiments. The salts were of the same degree of purity as those generally employed in the culture of protozoa and algae. The salts used in the preparation of artificial Huckleberry spring water apparently had no toxic effect.

Various investigators, notably Pringsheim (1930), have called attention to the deleterious effects produced on organisms by the kind of glass of which culture dishes are composed. He believed that unfavorable results were due to soluble constituents of the glass causing the medium to become more alkaline. Although ordinary finger bowls were used in the present experiments, no deleterious effect of the glass was observed. Successful cultures on fishmeal and fish muscle extract media were secured in the same glass finger bowls in which Uspenski's medium failed to support continuous growth and reproduction of *Volvox*.

Nor can failure to secure permanent cultures in the Uspenski medium be ascribed to an unfavorable pH. The initial pH was 7.6 (identical with that given by Uspenski and Uspenskaja) and remained at that value over a period of three weeks. That this is a favorable reaction is shown by the fact that the pH of freshly prepared fishmeal extract medium is 7.5–7.6, around which value it fluctuates from day to day gradually rising to a final pH of 8.0–8.2.

Mainx (1929) and Pringsheim (1930) secured rich cultures of *Volvox* in decoctions prepared from garden soil. Soils, however, vary widely in composition and reaction so that this method is not readily duplicable. Fishmeal medium, on the other hand, can be made up fairly accurately and is more readily reproducible. This is even more true of the fish muscle extract prepared with artificial Huckleberry spring water and the muscular tissue of the sunfish or butterfish.

The experiments of the present work show that the nutritional requirements of *Volvox aureus* may be more complex than is indicated by the work of Uspenski and Uspenskaja (1925) and that inorganic salts of spring water as well as organic substances of fish muscle are essential if this organism is to be maintained in culture for any length of time. What these salts and organic substances are, remains for further work to show; but it is quite possible that water-soluble growth promoting substances play an important rôle.

Summary

1. *Volvox aureus* Ehrenberg has been maintained in rich clone cultures for eighteen months without observable decrease in vitality on a medium prepared from spring water, commercial fishmeal, and ferric chloride.

2. The presence of iron in the medium was found to be essential to the continuous culture of *Volvox*. This was added as ferric chloride. Cultures lacking iron died out in about six weeks.

3. Either local spring water or Huckleberry spring water served satisfactorily in the preparation of fishmeal extract medium, but tap water and distilled water could not be substituted for these.

4. Spring water media containing peptone, beef extract, milk, or urine in various concentrations would not support continuous growth and reproduction of *Volvox*.

5. Uspenski's medium and modified Knop's solution were found to be of little value in the culture of *Volvox*.

6. Powdered muscle of the marine butterfish (*Poronotus triacanthus* Peck) or the fresh-water sunfish (*Lepomis gibbosus* Linnaeus), with lipoids extracted, could be substituted for commercial fishmeal, while skin and bone could not.

7. Spring water containing only the inorganic salts of fishmeal, or only the proteins, failed to support the culture of *V. aureus;* but spring water containing the cold water extractives and ferric chloride (one-half cc. of a one per cent solution of $FeCl_3 \cdot 6H_2O$/l.) gave excellent cultures which probably could have been carried indefinitely.

8. *V. aureus* could not be cultured in media containing lactic acid, urea, creatine, uric acid, or a combination of urea, peptone, and lactic acid in the several concentrations used.

9. A readily duplicable method for the culture of *V. aureus* is described.

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OXYGEN CONSUMPTION AND CARBON DIOXIDE ELIMINATION IN TETRAHYMENA GELEII FURGASON [1]

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Unicellular organisms make excellent material for the study of various cellular phenomena. *Tetrahymena geleii,* a colorless holotrichous ciliate, is an exceptionally desirable organism for such physiological studies, mainly because it is readily grown in rather simple, sterile organic media.

This organism contains cytochromes c, b, a, and possibly a_2 and its oxygen consumption is inhibited by cyanide and carbon monoxide (Baker and Baumberger, 1941). It grows well at ordinary oxygen tensions, but is most prolific in pure oxygen (Pace and Ireland, 1945). It soon dies in oxygen tensions below 10 mm. Hg partial pressure.

Many investigations have been carried out with *Tetrahymena* as the experimental organism. There is, however, much to be desired in respect to our knowledge concerning respiratory metabolism; knowledge that may lead to a better understanding of the respiratory mechanisms of cells in general. Since very little is known concerning oxygen consumption, carbon dioxide elimination, and respiratory quotient in *Tetrahymena,* the following investigations were made.

Material and Methods

The "W" strain of *Tetrahymena geleii,* kindly furnished by Professor George Kidder, used throughout these investigations, was grown in this laboratory in a 2 per cent proteose-peptone (Difco) solution. They grow very rapidly in this solution and usually reach maximum numbers within four to six days depending upon temperature and the number introduced.

A Barcroft-Warburg respirometer was used for ascertaining oxygen consumption and carbon dioxide elimination. Temperatures below room temperature were obtained in the bath by means of an Aminco refrigerating unit.[2]

Before each experiment the organisms were washed thoroughly in a buffered solution in which they were left during the experiment. This solution is one similar to that used by Pace and Belda (1944) for *Pelomyxa,* with slight modifications,[3] and was used for these tests chiefly because of the several advantages it has over the proteose-peptone solution. It is much simpler to work with since there is no

[1] Aided by a grant from the Penrose Fund of the American Philosphical Society.

[2] An extra set of manometers and shaking device for the Warburg outfit was purcheasd for this investigation by a grant-in-aid furnished by the University of Nebraska Research Council.

[3] The buffer solution used for washing and testing *Tetrahymena geleii* in the Barcroft-Warburg respirometer contained the following: $K_2HPO_4 \cdot 3H_2O$, 65.5 mg.; $NaH_2PO_4 \cdot H_2O$, 40.0 mg.; $CaCl_2$, 100 mg.; $MgCl_2$, 2.0 mg.; and redistilled H_2O to 1,000 ml.

food material on which bacteria can grow and therefore the dangers of contamination are minimized. Bacteriological technique was used throughout.

Usually, for each condition tested, two different ages of cultures were used: "young" cultures (three or four days) and "old" cultures (seven or eight days). These cultures were started by introducing several thousand organisms by means of a platinum wire loop into 70 ml. of sterile proteose-peptone solution in a 125 cc. pyrex erlenmeyer flask.

After washing and after proper dilution or concentration (by centrifugation) 5 ml. of culture solution of known population density were added to each Barcroft-Warburg flask except to the thermobarometer which contained 5 ml. of buffered solution without organisms.

Oxygen consumption and CO_2 elimination were determined by the direct method. For this purpose 0.2 ml. 10 per cent KOH was put into the inner wells (insets) of one-half the flasks and 0.2 ml. H_2O in the other one-half. To each of the onsets was added 0.3 ml. 3N H_2SO_4 to absorb any ammonia that might possibly be formed in metabolism. In every case this acid was dumped into the main compartment of the flask at the end of an experiment after which a reading was always made in order to account for the bound CO_2 which is released by the action of acid.

Results

Relation between population density and O_2 consumption and CO_2 elimination

Tetrahymenas were obtained from cultures of different ages. "Young" cultures were produced in the following manner: about $2{,}000 \pm 200$ tetrahymenas were added to 70 cc. 2 per cent sterile proteose-peptone solution in pyrex erlenmeyer flasks. These cultures were kept at room temperature ($24° \pm 2°$ C.) for 3 or 4 days when the organisms were washed in the buffer solution [3] by centrifugation. They were then ready for testing. Several different population densities were used, ranging from approximately 10,000 to 195,000 organisms per ml. Several experiments were conducted for each density.

In these experiments 5 ml. of fluid containing young tetrahymenas were added to each of six Barcroft-Warburg flasks (3 with KOH in inner well; 3 with H_2O). Observations and readings were made from time to time during the course of the experiment. Usually readings were made every hour, but in the greater population densities, they had to be made every 15 or 30 minutes; whereas, with some of the lower population densities several hours elapsed in some cases before a final reading was made. It depended entirely upon the rapidity of oxygen consumption at 25° C. After the reading was made at the end of an experiment, the acid in the side arm of each flask was dumped into the fluid containing the tetrahymenas. The shaking apparatus was turned on for 10 minutes, after which time another reading was made. The results of these tests are presented in Table I.

The oxygen consumption per organism, when young cultures were used, is greatest in densities of 13,000 to 33,000 organisms per ml. which were the lowest densities used, and least in the greatest densities. In other words, at least within these limits, oxygen consumption per organism is inversely proportional to population density. The respiratory quotient varied from 0.95 to 1.13 in all the different tests, except those in which the lowest population densities were used where the R.Q. averaged 1.41.

The "old" organisms were produced in the same way as the "young" except that they were not used until 7 or 8 days after starting the cultures. The same procedures were followed as in the previous tests. The population densities varied between 12,000 and 265,000 organisms per ml. These results are also presented in Table I.

TABLE I

Oxygen consumption, carbon dioxide elimination and respiratory quotient in "young" and "old" cultures of Tetrahymena geleii *with different population densities. Temperature 25° C.; average volume of one million organisms = 23.6 mm.³; 42,370 organisms equal to one cubic millimeter.*

No. of organisms per ml.	Age of culture in days	No. of tests	O_2 consumption in mm.³ per hr. per million organisms	O_2 consumption in mm.³ per hr. per mm.³ cell substance	CO_2 elimination in mm.³ per hr. per million organisms	CO_2 elimination in mm.³ per hr. per mm.³ cell substance	R.Q.
Organisms from "young" cultures							
13,000–19,000	3	6	372	15.7	526	22.3	1.41
30,000–33,000	3	9	372	15.7	356	15.0	0.95
41,500–51,500	4	6	325	13.7	350	14.8	1.07
88,000–98,000	3	6	322	13.6	328	13.9	1.01
107,600–121,000	3½–4	9	252	10.6	249	10.5	0.99
195,000	3½	3	246	10.4	278	11.7	1.13
Organisms from "old" cultures							
12,000–12,600	7–8	9	198	8.4	221	9.3	1.11
24,000–27,500	7–8	6	223	9.4	258	10.9	1.15
38,000–39,400	7–8	9	204	8.6	247	10.4	1.21
69,000–72,600	7	6	278	11.7	357	15.1	1.28
87,600–95,000	7–8	6	217	9.2	263	11.1	1.21
135,000–148,700	7	9	207	8.7	240	10.1	1.15
213,000–265,000	7	6	230	9.7	272	11.5	1.18

The oxygen consumption varies from one population density to another much more so than in the "young" cultures, but there is a definite increase in consumption up to approximately 70,000 organisms per ml. and then a decrease with further increase in numbers per unit volume. Carbon dioxide elimination was also ascertained and the R.Q. was found to be greater than unity in every case where old cultures were used. The R.Q. increased progressively from 1.11 when approximately 12,000 organisms per cc. are used, to 1.28 when approximately 70,000 are used. It then decreases with further increase in cell population until it is 1.18 in cultures containing between 213,000 and 265,000 organisms per ml.

Relation between temperature and oxygen consumption

Tests were made to ascertain the oxygen consumption and carbon dioxide elimination in Tetrahymena at different temperatures. The temperatures used varied from 10° to 35° C. (with 5° increments). As in the previous experiments tests were run on both "young" and "old" organisms. The results are given in Table II.

There is an increase in oxygen cousumption as temperatures increase up to 25° C. Above 25° C. the O_2 consumption decreases directly with increase in temperature. This applies to organisms obtained from both young and old cultures. Attempts were made to ascertain oxygen consumption at 40° C. but the organisms died at this temperature. The carbon dioxide elimination and hence the R.Q. values were high in all the tests made. The latter ranged between 1.05 and 1.39.

TABLE II

The effect of temperature on oxygen consumption, carbon dioxide elimination and respiratory quotient in Tetrahymena geleii. *Average volume of one million organisms, 23.6 mm.³; 42,370 organisms equal to one cubic millimeter.*

Temperature, ° C.	No. of organisms per ml.	Age of culture in days	No. of tests	O_2 consumption in mm.³ per hr. per million organisms	O_2 consumption in mm.³ per hr. per mm.³ cell substance	CO_2 elimination in mm.³ per hr. per million organisms	CO_2 elimination in mm.³ per hr. per mm.³ cell substance	R.Q.
Organisms from "young" cultures								
10	31,800	3	3	50	2.1	57	2.4	1.14
15	52,900–67,500	3–3½	9	125	5.3	142	6.0	1.13
20	38,750–41,160	3	6	193	8.1	227	9.6	1.17
25	41,500–51,600	4	6	325	13.7	350	14.8	1.07
30	32,000–60,000	3	6	227	9.6	297	12.5	1.30
35	10,000	3	3	215	9.1	287	12.1	1.33
Organisms from "old" cultures								
10	54,000	8	3	25	1.0	35	1.5	1.39
15	53,000–73,300	7	12	92	3.9	91	3.8	0.99
20	65,500	6–7	6	128	5.4	135	5.7	1.05
25	69,000–72,600	7	6	278	11.7	357	15.1	1.28
30	45,000–50,800	7	6	228	9.6	285	12.0	1.25
35	16,800–19,160	8	6	254	10.7	287	12.1	1.12

O_2 consumption and CO_2 elimination in Tetrahymena in proteose-peptone solution

All the previous tests which have been reported here were made on Tetrahymena in the buffer solution given in footnote 3. None had been made on organisms in solutions in which they had grown and lived, i.e., proteose-peptone solution. The following experiment was carried out in order to make these determinations. Thus, the tests were conducted in the same way as the preceding ones, except that the organisms (1) were kept in the solution in which they had grown (2 per cent proteose-peptone) or (2) were washed in fresh proteose-peptone and then put into the manometer flasks in this solution. It was found that the hydrogen-ion concentration did not hold to a constant value as well as in the buffer solution but the change did not seem to harm the organisms (the greatest change noted was a change from pH 6.8 to pH 7.3). The results are presented in Table III.

In the tests in which "young" cultures of Tetrahymena were used in fresh proteose-peptone solution, the oxygen consumption proved to be much greater than in those tested in buffer solution (at 15° C., 271 mm.³ compared to 125 mm.³ per hour

per million organisms in buffer solution; at 25° C., 666 mm.^{3} compared to 325 mm.^{3}). In the organisms from "old" cultures, however, the oxygen consumption was somewhat lower than that in buffer solution (at 15°, 70 mm.^{3} as compared to 92 mm.^{3}). Variance in numbers, however, must be taken into consideration.

TABLE III

Oxygen consumption, carbon dioxide elimination and respiratory quotient in Tetrahymena geleii *in 2 per cent proteose-peptone solution. Temperature varied as indicated; average volume of one million organisms, 23.6 mm.^{3}; 42,370 organisms equal to one cubic millimeter.*

Temperature, ° C.	No. of organisms per ml.	Age of culture in days	Condition of proteose-peptone solution	No. of tests	O_2 consumption in mm.^{3} per hr. per million organisms	O_2 consumption in mm.^{3} per hr. per mm.^{3} cell substance	CO_2 elimination in mm.^{3} per hr. per million organisms	CO_2 elimination in mm.^{3} per hr. per mm.^{3} cell substance	R.Q.
15	30,400	3	fresh	4	271	11.4	328	13.9	1.21
15	32,060–40,000	7	old	6	70	2.9	197	8.3	2.81
25	25,800	3	fresh	3	666	28.2	849	35.9	1.27

A very interesting observation brought out by these results, is that in all tests, the R.Q. is considerably above 1.0. This is especially so in the case of the "old" organisms where the average R.Q. for all tests was 2.81.

Significance of the high respiratory quotients

The question arises as to the true meaning of these high respiratory quotients. In all but a few experiments covered by this report, they are greater than 1.0. It is generally known that high values for R.Q. may be obtained chiefly under two conditions: (1) when fat is in the process of formation in cells or tissues, or (2) when it is necessary for the cell or tissue to go into oxygen debt, temporarily at least, during which time the ratio between CO_2 eliminated and O_2 consumed would be much greater than usual.

Tetrahymena produces fat in the form of small globules located in the anterior half of the organism. Observations were made upon these organisms under various conditions in order to ascertain whether or not respiratory quotients varied and if so, whether or not fat content varied also. As far as could be seen by the methods used there was no difference in fat content in organisms under different conditions even though the respiratory quotients varied considerably.

A few tests were made in an attempt to ascertain the oxygen consumption and carbon dioxide elimination of *Tetrahymena* in nitrogen. The nitrogen used, however, was a commercial grade and hence traces of oxygen were present. Although the results obtained by these tests were not conclusive, they indicate that these organisms are anaerobic, at least to a limited extent, thus confirming the results of Thomas (1942). The respiratory quotients calculated from the results ranged between 1.49 and 2.87.

DISCUSSION

Hall (1938, 1941), Baker and Baumberger (1941) and Ormsbee (1942) have studied respiration in *Tetrahymena geleii*. The results of these several investiga-

tions appear to differ, in some cases, considerably but when they are analyzed carefully the differences, for the most part, may be explained.

Some of the results of the present investigation are in close agreement with those of the aforementioned workers. This is especially true, when our results are compared with those of Ormsbee (1942) in which he found that the O_2 consumption for these organisms in 2 per cent proteose-peptone solution is 632.5 mm.^{3} per hour per million at 26.8° C.; in our studies, it was found that the O_2 consumption in 2 per cent proteose-peptone is 666 mm.^{3} at 25° C. The results obtained in non-nutrient media should not be compared since the salts differ greatly in the solutions used by different investigators, but it is found that even in these cases there is some agreement, especially when all the factors are taken into consideration. An explanation of some of the differences, for example, might be found in the fact that different population densities were used. According to our results, as the density of population increases the rate of oxygen consumption per individual decreases. This observation, along with possible differences in age of cultures, and also the difference in culture media before and during the experiment, could account for the slight difference in oxygen consumption as noted in these reports. This age difference is especially noticeable when the organisms are tested in the proteose-peptone solution in which they were grown. There is not only an inhibition of division with age but also a great decrease in oxidative metabolism.

Thomas (1942) showed definitely that *Tetrahymena* is adapted, at least partially, to an anaerobic existence, recovery being dependent upon oxidative metabolism. He believes the processes involved may be similar to those of mammalian striated muscle. Pace and Ireland (1945) showed that *Tetrahymena* can live and grow at very low oxygen tensions but that its growth is best at high tensions. This organism does not grow or live for any great length of time in the total absence of oxygen. Results presented in this report confirm those of Thomas in this respect.

The respiratory quotients obtained for *Tetrahymena* were all high under the conditions in which they were determined. They were highest of all in those organisms confined in practically pure nitrogen. Carbon dioxide is produced in large quantities. It is possible that the metabolism is similar to that of vertebrate striated muscle. The extent of anaerobiosis is limited for *Tetrahymena* just as it is for muscle. *Tetrahymena* can live for several days in the absence of oxygen but soon dies unless oxygen is added. Specht (1934) found the same to be true for *Spirostomum*. He obtained high R.Q. values when this organism was exposed to low O_2 tensions. Many protozoan forms function in the same way (von Brand, 1946).

Summary

1. Oxygen consumption and carbon dioxide elimination of *Tetrahymena geleii* has been ascertained for different temperatures, for different population densities, and for "young" and "old" cultures.

2. The tests were conducted in most of the experiments with washed organisms in inorganic buffer solution.

3. When "young" organisms are used, the oxygen consumption per unit volume of cell substance is inversely proportional to population density. The respiratory quotients for all tests were nearly always above 1.0.

4. When "old" organisms are used there is an increase in O_2 consumption per unit volume of cell substance with an increase in population density up to 69,000–

72,600 organisms per ml. Densities above this result in lower consumptions. Respiratory quotients varied from an average of 1.11 in low population densities to 1.28 at optimum densities.

5. Oxygen consumption is always greater in the "young" cultures than in the "old" cultures, densities being equal.

6. With an increase in temperature, the O_2 consumption increases to a maximum at 25° C. in both "young" and "old" cultures. Above this temperature the consumption decreases. In all tests, from 10 to 35° C., R.Q. values were above 1.0.

7. "Young" organisms, tested in fresh solution of the same kind in which they were grown, 2 per cent proteose-peptone (Difco), show much greater O_2 consumption than those tested in inorganic buffer solution. "Old" organisms, however, tested in the same solution in which they had grown, have a lower O_2 consumption than old specimens tested in fresh inorganic buffer solution.

8. Very high respiratory quotients were obtained for the organisms in proteose-peptone, especially in "old" cultures where the average was 2.81.

9. In nitrogen gas with but minute traces of oxygen, *Tetrahymena* utilizes the small quantity present and gives off comparatively large quantities of carbon dioxide. The R.Q. values are extremely high.

10. *Tetrahymena geleii* is to a limited extent anaerobic but it can live only with difficulty for more than a few days in the absence of oxygen.

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INFLUENCE OF TEMPERATURE ON THE ASPHYXIATION OF YOUNG GOLDFISH (CARASSIUS AURATUS L.) UNDER VARIOUS TENSIONS OF OXYGEN AND CARBON DIOXIDE

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Introduction

In their preliminary work on the sensitivity of Ontario fishes to carbon dioxide as measured by the asphyxial tension of oxygen of fish in sealed jars containing various concentrations of dissolved carbon dioxide, Fry and Black (1938) observed a difference in the response of two sample groups of the minnow, *Chrosomus eos*. The group taken in the autumn from a cold stream near Toronto was found to be appreciably more sensitive than the group from a bog lake in Algonquin Park at the height of the summer warming. These data were subsequently published in a review (Fry, 1939). At that time there was no indication as to the cause of this difference since the two groups came not only from different parts of the country but also from different habitats.

Further measurements were made in Algonquin Park in 1939 when the work was continued into early fall. Some comparisons were made of curves showing sensitivity to carbon dioxide for the same species from the same locality at different times of year. The same effect was noted as for *Chrosomus eos,* although the data were rather scanty. Observations of the same sort were made again in 1940 and, in particular, material was gathered for the brown bullhead (*Ameiurus nebulosus*). The results are illustrated in Figure 1. There is an increased sensitivity in late summer or early fall when the water is cooling. These tests were carried out in water of the same temperature as that in which the animals were captured.

Reasoning by analogy from the fact that cold water species such as the trout fail to take up oxygen in the presence of much lower tensions of carbon dioxide than do warm water species such as the brown bullhead, it was concluded that probably there would be some such trend within each species and that concomitant with the process of thermal adaptation there might be a change in respiratory sensitivity in the direction observed.

Meanwhile an opportunity to test this experimentally had presented itself in February 1939, when two of the authors in collaboration with Professor Laurence Irving and using the facilities of the experimental hatchery at Cornell University, measured the respiratory sensitivity of the speckled trout at 1° C, the temperature of the hatchery water at that time. The respiratory sensitivity was also measured of a group of these fish which had spent 24 hours at 13° C. The sensitivity of the group subjected to the warm water for even that short period of time was appreciably decreased.

This experiment, however, was only of a preliminary nature and at that time it was not possible to carry it to its logical conclusion. For this reason we undertook to do a further series of experiments to determine the effect of temperature on the respiratory sensitivity of thermally adapted goldfish. The temperatures chosen covered the biokinetic range of temperature for this species. Goldfish were maintained at each temperature level for a period of time quite sufficient to ensure a thorough thermal adaptation. The goldfish was chosen because of its suitability as a laboratory animal, because the thermal tolerance for the young goldfish had been

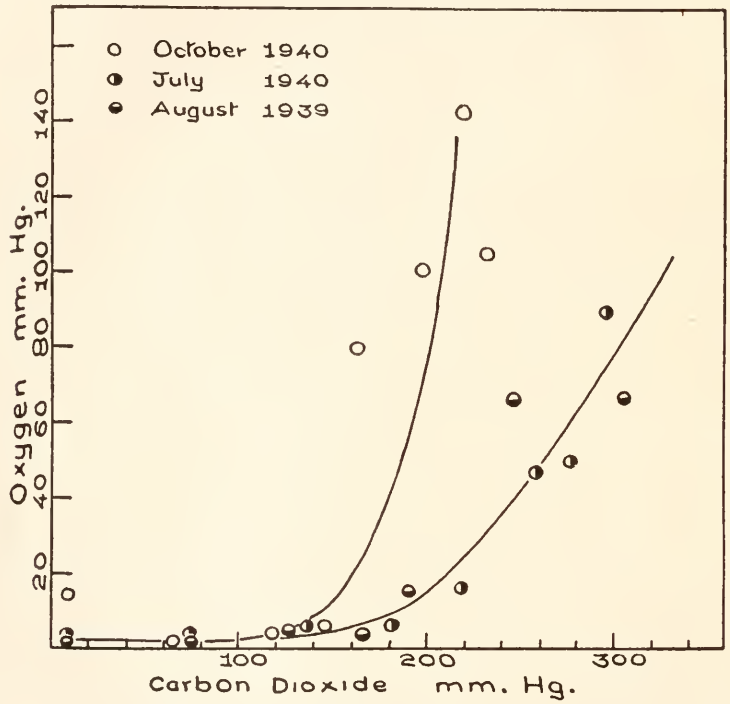

FIGURE 1. Seasonal variation in the extent to which the brown bullhead can reduce the oxygen content of water in sealed bottles in the presence of various tensions of carbon dioxide. In July and August the fish were acclimated to approximately 20° C., in October to 13° C. They were tested at the acclimation temperatures in each instance.

plotted with some exactitude (Fry *et al.*, 1942), and also because the rate of thermal adaptation of goldfish was being measured (Brett, 1946) at the time these experiments were begun.

MATERIAL AND METHODS

The goldfish, *Carassius auratus* (Linnaeus), were the ordinary long finned commercial variety of goldfish purchased from commercial dealers in Ontario. They were approximately one year old, of both sexes, and varied from 2½ to 4 inches in length. The fish were fed fox chow during the period of acclimation.

A group of fish was acclimatized to one of the following temperatures: 1° C., 7° C., 15° C., 20° C., 25° C., and 32° C. The overall allowance for acclimation of each

group of fish from 7° to 32° was 1° C. or less per day until the desired temperature was reached. Since complete acclimation to low temperatures takes a longer time (Doudoroff, 1942) the fish used for the 1° C. curve were put from a 7° C. tank into a 1° C. tank and left for two months.

When acclimation was complete each fish was put in a bottle of water (275 ml. capacity) of the same temperature and containing a known tension of carbon dioxide and at least 150 mm. tension of oxygen. During the experiment the sealed bottles containing the fish were kept in a water bath at the temperature of acclimation. When all respiratory movements of the fish had ceased the water in the bottle was analyzed for free carbon dioxide and oxygen. The tension of carbon dioxide was determined as follows: a small bubble of air (0.1–0.3 ml.) was equilibrated with 35 ml. of the water for five or more minutes at the temperature of the experiment at room pressure; the bubble was then transferred to a micro-gas-analyzer (after Krogh, 1908) and the carbon dioxide content of the gas phase was found by absorption of carbon dioxide with $\frac{1}{4}$N potassium hydroxide. The tension was obtained by multiplying the fraction of carbon dioxide in the gas phase by the barometric pressure minus the vapor pressure of the water at the temperature of the experiment.

Dissolved oxygen was determined by the unmodified Winkler method, using a 50 ml. sample of water. The pressure of oxygen was calculated by relating the quantity of oxygen found to the solubility at the temperature of the experiment and multiplying the fraction obtained by 760.

Results

The data for the experiments at each of the six temperatures (1° C., 7° C., 15° C., 20° C., 25° C., and 32° C.) are given in Table I and illustrated in Figure 2. To

Table I

The extent to which goldfish acclimated to various temperatures can reduce the oxygen content of water containing dissolved carbon dioxide. Each pair of values is the mean of three to five determinations

1° C.		7° C.		15° C.		20° C.		25° C.		32° C.	
CO_2 mm. Hg	O_2 mm. Hg	CO_2 mm. Hg	O_2 mm. Hg	CO_2 mm. Hg	O_2 mm. Hg	CO_2 mm. Hg	O_2 mm. Hg	CO_2 mm. Hg	O_2 mm. Hg	CO_2 mm. Hg	O_2 mm. Hg
20	5.5	27	3.5	17	2.9	34	7.5	25	4.7	14	8.0
64	4.9	79	3.5	66	3.5	88	8.3	141	13	144	8.0
87	48	100	5.8	110	5.9	120	2.9	172	7.6	168	7.0
98	24	113	16	131	12	139	7.3	200	42	186	7.3
111	46	128	38	144	49	154	41	224	120	212	46
141	66	138	66	153	49	166	25	286	272	226	102
				171	74	197	80			240	216
				192	75	242	139				
				237	163						

reduce the spread the original figures have been grouped into averages of 3 to 5 determinations. This averaging was carried out by arranging all the values in a particular series in order of the magnitude of the carbon dioxide tension and then averaging the values for consecutive groups.

The course at each temperature appears to be as follows. Up to a certain tension of carbon dioxide the oxygen in the bottle is reduced to a uniform and low level before death of the fish ensues. Above a certain carbon dioxide tension the level of the carbon dioxide influences the level to which the oxygen can be reduced, i.e., the greater the carbon dioxide content the higher the level of oxygen in the water at

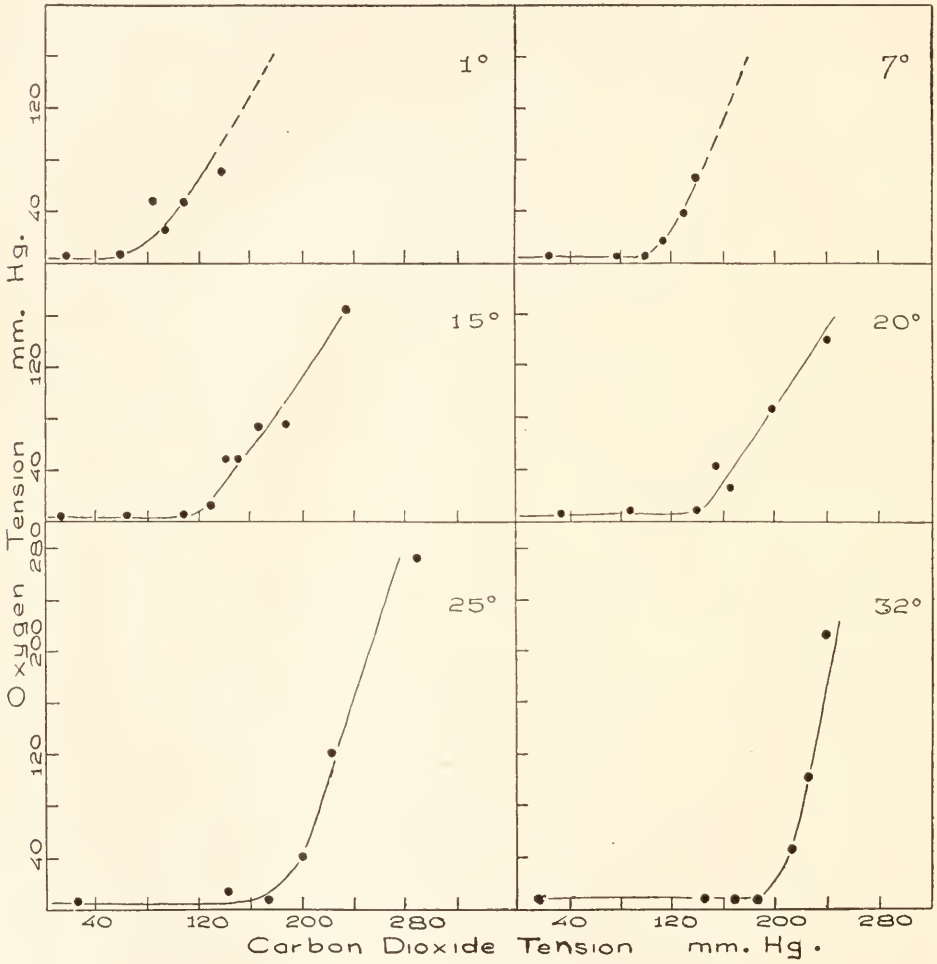

FIGURE 2. The extent to which goldfish can reduce the oxygen content of water in sealed bottles in the presence of dissolved carbon dioxide at various temperatures of acclimation. Each point is the average of three to five determinations.

asphyxiation. As the acclimation temperature is increased the level of carbon dioxide required to limit the level to which the oxygen is used also increases. There is some indication that the rising portion of the curve becomes steeper at the higher acclimation temperatures.

The data from the six curves in Figure 2 are brought together in Figure 3 where the change in sensitivity, i.e., the number of arbitrary units contained within

each curve, is plotted against the temperature of acclimation. The sensitivity of the fish seems to decrease almost directly with the increase in temperature of acclimation.

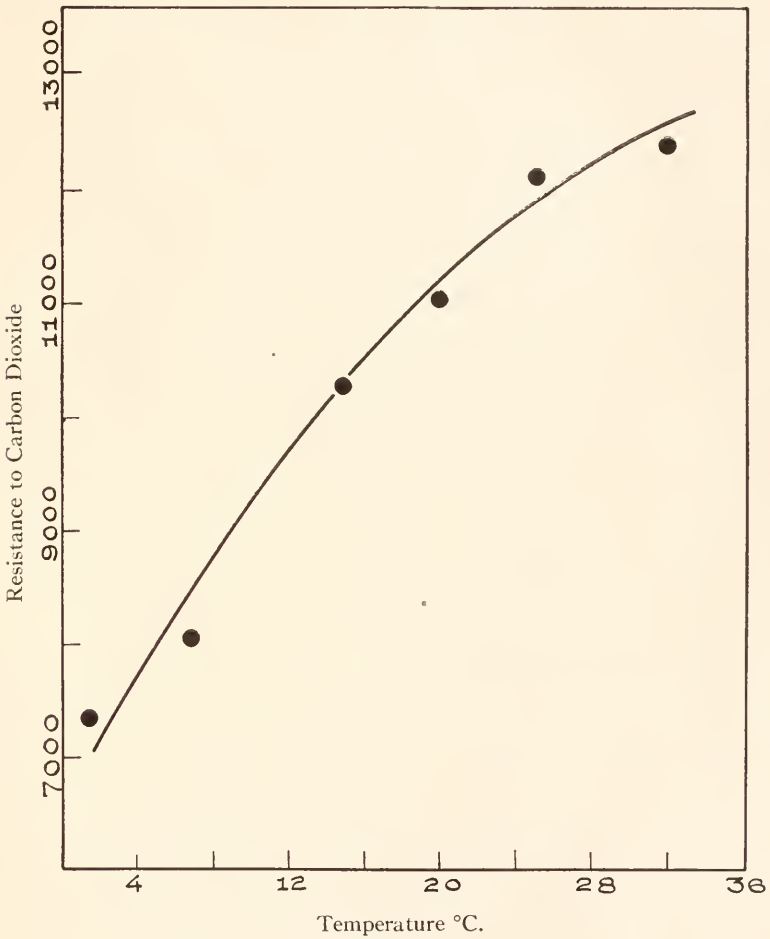

FIGURE 3. The relation between utilization of oxygen in the presence of dissolved carbon dioxide and the temperature to which goldfish are acclimated. The units of resistance to carbon dioxide given are the areas composed by oxygen × carbon dioxide (as mm. Hg) between the resistance curves in Figure 2 and a horizontal line drawn across at 160 mm. Hg oxygen (air saturation). Sensitivity to CO_2 referred to in the text is a reciprocal function of resistance.

DISCUSSION

The ability of the fish to use oxygen in the presence of carbon dioxide as measured here, varies considerably in goldfish adapted to various temperatures within their biokinetic range. The difference between goldfish acclimated to water of 1° C. and those acclimated to water of 32° C. is of the same order as the differences between the common sucker (*Catostomus commersonnii*) and the brown bullhead

(*Ameiurus nebulosus*) when these species are acclimated to about 20° C. (Fry, 1939).

The change in the respiratory sensitivity attendant on change in the environmental temperature is such that when goldfish are adapted to low temperatures their respiratory sensitivity tends toward the type found in cold water species (common sucker): when adapted to high temperatures the respiratory sensitivity resembles that of warm water species (brown bullhead).

Other changes in the respiratory metabolism of fish have been found to be attendant on changes in thermal adaptation (Wells, 1935; Sumner and Wells, 1935; Sumner and Lanham, 1942; Sumner and Doudoroff, 1938). Indeed Sumner and Doudoroff used the change in resistance time to cyanide poisoning as an index of change in thermal adaptation. The change shown in our work appears however to be unique in that the absolute level of respiratory sensitivity is lower at a higher temperature.

While no definite conclusions can be drawn it is interesting to speculate on the change of organization which could bring about this change in respiratory sensitivity. Two phases of the circulatory systems of fish have been shown to be correlated with their respiratory sensitivity. Black (1940) noted that there were significant differences between four species of freshwater fish and the effect of carbon dioxide on the affinity of hemoglobin in whole blood for oxygen, and further, that the fish with the greater sensitivity to carbon dioxide are found in colder habitats. Hart (1945) showed a similar correlation between the stroke output of the heart and the respiratory sensitivity of a series of species. He also pointed out that the influence of the mechanical factor in respiratory transport may at times be so great as to displace the order of respiratory sensitivity of species which might be expected from a comparison of the chemical characteristics of their oxygen transport system. In addition to Hart's material another probable instance of this is found in the marine fish, the tautog (*Tautoga onitis*) and the toadfish (*Opsanus tau*). The respiratory tolerance of these fish is quite different, the toadfish being able to withstand up to 250 mm. of carbon dioxide whereas the tautog can only tolerate 150 mm. (Safford, 1940). The chemical properties of the blood of these fish for transporting oxygen in the presence of carbon dioxide are, however, very similar (Root, Irving, and Black, 1939).

Thus the change in respiratory sensitivity with change in temperature as displayed by goldfish adapted to their thermal environment may be a reflection of changes in either the mechanical or chemical aspects of their circulatory system and the problem remains to see to what extent each may be acting.

There is another change in the organism that may influence the measure of respiratory sensitivity as determined here for the goldfish. The areas enclosed above and to the left of the carbon dioxide-oxygen curves in Figures 1 and 2 are not entirely areas of tolerance in the sense that the animal could remain alive indefinitely under conditions represented by any combination of oxygen and carbon dioxide within these areas. At points in the marginal area near the limiting curve the animal is in a dying condition and its ability to transport oxygen will not depend on its tolerance towards those conditions but upon the length of time its organization can resist the ultimate breakdown. During the part of this period when the ventilatory and circulatory systems are still functioning, sub-minimal amounts of oxygen will be transported to the tissues of the dying animal. While these amounts of oxygen may be sub-minimal from the point of view of maintaining the life of the organism they

may be quite significant in extending the carbon dioxide-oxygen curve considerably beyond the region of tolerance. This effect was well shown by Wiebe *et al.* (1934) who demonstrated that after a fish introduced into a sealed container had extracted oxygen in the presence of a certain pH until it had died, a second subject introduced into the same container could reduce the oxygen still further by a significant amount before it also died.

Thus the decrease in the respiratory sensitivity at higher temperatures as measured by asphyxiation in a sealed bottle could in part be accounted for by increase in resistance of the tissues to oxygen lack or carbon dioxide excess, taking resistance to mean the length of time that an organism can resist a lethal condition before it finally succumbs. However one would not expect this factor to account for anything like the whole of the change in respiratory sensitivity observed.

When conditions are standard with respect to heredity and environment the respiratory sensitivity curves appear to offer a valid physiological yardstick whatever question there may be of exactly what these experiments measure. In the present series of experiments for instance the 20° curve was obtained in 1945 using material of the same age and purchased from the same source as that which was used for the determinations at the other temperatures in 1943, and as will be seen the data are quite consistent.

Necessity for complete acclimation to secure standard results becomes apparent when temperature effect over the biokinetic range at one level of thermal adaptation is considered. We have made two comparisons on fish acclimated to 20° C. In one instance the experiments were conducted in water at 12° C., in the other at 28° C. These data are given in Table II and may be compared with the values given for

TABLE II

The extent to which goldfish acclimated to 20° C. can reduce the oxygen content of water containing dissolved carbon dioxide at 12° C. and at 28° C. Each pair of values is the mean of four determinations

Acclimated to 20° Experiments at 12°		Acclimated to 20° Experiments at 28°	
CO_2 mm. Hg	O_2 mm. Hg	CO_2 mm. Hg	O_2 mm. Hg
24	5.7	28	7.9
82	5.4	69	7.4
100	8.6	121	11
111	9.8	140	18
127	19	157	60
152	32	169	25
181	100	186	141
		217	184

20° C. in Table I. In both cases the respiratory sensitivity proved to be greater than that measured at the temperature of acclimation. The degree of difference that these results show is somewhat surprising in view of the fact that the difference in temperature at which the experiments were carried out was only 8 degrees in either direction from the acclimation temperature, while at the acclimation level chosen the lower lethal temperature is about 18° C. below and the upper lethal 15° C. above the acclimation temperature.

Summary

1. By asphyxiating goldfish in sealed bottles of water containing dissolved carbon dioxide and oxygen, it was found that the ability of the fish to use oxygen in the presence of carbon dioxide increases as the acclimation temperature of the fish is raised.

2. Goldfish acclimated at 1° C. are limited in their ability to use oxygen when a tension of 60 mm. or more carbon dioxide is present in the water, whereas goldfish acclimated at 32° C. are not limited in the utilization of oxygen unless the carbon dioxide tension is 200 mm. or more. Fish at intermediate temperatures of 7°, 15° 20° and 25° C. begin to show a decrease in the ability to use oxygen when they are in the presence of carbon dioxide tensions of 100, 120, 140, and 170 mm. respectively.

3. Reliable results could be obtained only after complete acclimation of the fish to the temperature at which the experiments were made. However, with acclimated fish of similar stock the result could be reproduced from year to year at the same season.

4. The explanation for this change in sensitivity to carbon dioxide in acclimated fish lies possibly in the effects of temperature on both the mechanical transport of the blood and the chemical transport of oxygen by the blood.

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